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INITIAL BLOOD STORAGE EXPERIMENT

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EXPERIMENT (Center for Blood Research)
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The Center for Blood Research
Contractor

Subcontractors

Arthur D. Little, Inc.
The Children's Hospital
Lahey Clinic Medical Center
University of Massachusetts Medical Center

INITIAL BLOOD STORAGE EXPERIMENT

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SPACE SHUTTLE BLOOD STORAGE EXPERIMENT¹

ADDENDUM

The Center for Blood Research
Children's Hospital
Lahey Clinic
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Arthur D. Little, Inc.

7.0 ADDENDUM

7.1 Introduction

This addendum provides further justification for the use of microgravity for the Blood Storage Experiments. The addendum is comprised of five portions: 1) significance, 2) working hypothesis, 3) storage lesions at earth's gravity, 4) biomaterials and 5) summary.

7.2. Significance

The possibility of conducting experiments with the formed elements of the blood under conditions of microgravity opens up important opportunities to improve our understanding of basic formed element physiology as well as contribute to improved preservation of the formed elements for use in transfusion. In attempting to identify the possible effects of reduced gravitational attraction on formed elements during preservation, it is essential to recognize that 1) the formed elements - red cells, white cells and platelets - are complex living systems which depend upon multiple interactions with their extracellular environment, of which gravitational force is but one component; and 2) the survival within the circulation and the functional capacity of stored formed elements after transfusion are markedly diminished when compared to cells from fresh blood.

Multiple factors affect blood cells during storage such as sedimentation, diffusion of nutrients and metabolites, gas transport, mechanical trauma due to mixing and leaching of chemical compounds from the container material. Storage under standard blood bank conditions invariably exposes blood to Earth's gravity, and leads to injuries, i.e. storage lesions (see below) to the stored formed elements.

Exposure of the blood at microgravity in the space shuttle is expected to result in a greatly reduced perturbation of cell functions and permit the separation of sedimentation, mixing and container lesions which are associated with terrestrial blood storage.

Information gained from storage experiments under microgravity as proposed will add a new and important dimension to our understanding of blood cell membrane functions and the storage defects, and serve as a guideline for design of the improved methodology for blood storage on Earth. Further, the knowledge gained

can serve as a guide not only for the polymer industry to develop alternative blood bags but also for the pharmaceutical industry to study drug effects on cell membranes.

7.3

An Experimental Hypothesis

The objective of this proposal is a comparative evaluation of the physiological, biochemical and physical changes of the membrane of the erythrocyte, platelet, and leukocyte during storage under two specific conditions: standard blood bank conditions and microgravity, utilizing three plastic formulations.

Given the complexity of living biological systems, the wide range of possible measurements which could be made and the limited set of measurements which have been chosen in the first set of experiments, the following are some of the results we expect from our studies:

Studies comparing the state of human blood following prolonged preservation at a) Earth's gravity, and b) the microgravity within the shuttle, should shed important new light on the fundamental cell physiology of each type of blood cells, and, if we are fortunate in our choice of measurements, should provide significant new clues to improved preservation of these important materials on earth.

For cells preserved at microgravity, the adverse effects of sedimentation and mixing should be less apparent against the background of container lesions and aging lesions.

Red cells preserved at microgravity should exhibit improved pH control and more nearly physiological levels of important metabolites such as ATP and 2,3 DPG.

Platelets and leukocytes which are more sensitive than red cells to harmful effects from cell-cell interactions, should show superior indices of cell viability and potential to function after storage at microgravity in comparison to controls kept on Earth.

Adverse effects of synthetic polymers which originate from cell-surface interactions should be reduced during microgravity preservation.

7.4 Storage Lesions at Earth's Gravity

In this portion, the discussion is divided into four sections: 1) storage lesions overview, 2) red cell storage on Earth, 3) platelet storage on Earth, and 4) leukocyte storage on Earth.

7.4.1 Storage Lesion Overview

In discussing the science of blood formed element preservation, it has proved useful to classify observed results into several categories of effects, termed lesions. It must be noted, however, that this does not necessarily mean that we can identify a specific area of physical damage or injury with each lesion.

7.4.1.1 Sedimentation Lesions

The sedimentation lesion results from the dense packing of the formed elements, with resulting intensity of injurious cell-cell and cell-container surface interactions. Under these circumstances, metabolic injury can occur as cells are deprived of their substrates or as cell wastes concentrate in the immediate vicinity rather than being dissipated and removed as in the physiological state.

Within the blood stream, erythrocytes, leukocytes and platelets, like all cells, are metabolically active. They are supplied with energy in the form of small organic molecules (substrates), which are contained in the fluid medium, the plasma, which surrounds them, and they depend upon the plasma to accept the waste products of their metabolism. If these waste products, particularly carbon dioxide, are not removed, they can quickly alter the extracellular environment and injure the cell.

The blood cells utilize energy to carry out their physiological functions, and to maintain their own integrity. Thus, the red cell requires a constant supply of energy derived primarily from the metabolism of glucose to maintain its unique biconcave discoid shape. If the metabolic system fails, the cells invariably die.

The rationale of preservation of the red blood cell, the formed element about which we know the most, takes advantage of what is known about red cell metabolism. A preservative diluent solution provides glucose, the main energy source, and other chemical intermediates needed

to foster a viable metabolic system. Another constituent of the diluent lowers the pH of the red cell suspension. And finally, and most important, the temperature is reduced from 37.5°C to between 1 and 6°C. Under these conditions, the metabolic system is put into a quasi-hibernative state, in which it survives remarkably well. Glucose utilization proceeds at a very slow rate, as does carbon dioxide production; and no oxygen is required.

It follows that an experiment at microgravity will shed light on cellular metabolism and other parameters of cellular viability which are most sensitive to the harmful effects of gravitational force.

7.4.1.2

Aging Lesions

Aging lesions arise from the simple fact of biologic aging which all biological cells are subject to. Red Cells, for example, normally live for 120 days in the circulating blood. Thus, if preserved outside the body, red cells may continue to age. In the blood bank, at 1-6°C, this aging contributes to the time limit for preservation. The specific cause of biologic aging is not known but is thought to include membrane changes of cells.

The ability of blood cells (red cells, platelets and leukocytes) to maintain their size and shape against the stress of gravity requires the expenditure of energy. Reduction or elimination of this stress in microgravity will lead to marked energy conservation. As a result, the characteristics of the aging processes of blood cells can be better defined.

7.4.1.3

Container Lesions

Container lesions are defined as those effects brought about by exposure of the blood or its formed elements to foreign surfaces such as glass tubes, metal needles and plastic bags. These include but are not limited to cell-foreign surface effects and cell-foreign surface constituent effects. A whole field of endeavor is concerned with cell-foreign surface effects; these being particularly important in respect to implanted prosthetic devices, such as artificial heart valves, prosthetic aortas, etc. In these cases, biocompatibility is probably determined by physical forces at the interface between the solid and liquid phases of the blood forces which may adsorb and rupture formed elements, or which may damage the complex composition of lipids and proteins in the cell membrane.

An example of a cell-foreign surface constituent effect is the leaching of the organic chemical plasticizer, DEHP, from the plastic blood bag onto the red cell surface.

7.4.1.4

Mixing Lesions

Mixing lesions represent the fourth type of storage lesions which are encountered in blood preservation. These result from the use of mixing procedures to improve the preservation of separated formed elements. In a simplistic view, mixing is usually used to prevent the deleterious effects of sedimentation.

Mixing ostensibly reduces the deleterious effects of "crowding" of cells, results in the accessibility of essential nutrients to the cell, and eliminates the undesirable effects on the cell of accumulated waste products.

But mixing unfortunately can introduce undesired effects as well. Mixing subjects cells to energetic forces of turbulence, cell-container surface and cell-cell interactions. Not surprisingly, the mixing can produce variable effects which are often brought to light when mixing is carried out in different polymer bags and modes of movement (see Table I in section 7.4.3.1.).

Microgravity which maintains the cells in suspension, provides a unique way of avoiding both the sedimentation and the mixing lesions.

7.4.2

Red Cell Storage on Earth

7.4.2.1

Sedimentation Effects on Red Cell Storage

Storage of whole blood under standard blood bank conditions leads to sedimentation of the cells which produces storage lesions because metabolites accumulate around the cells, pH decreases, and substrates are depleted. These lesions develop as a result of the limitation of diffusion which in turn is determined by the thickness of sedimented cell layers.

Recently, Beutler et al. (1) compared 24-hour post-transfusion survival and hemolysis of red cell concentrates collected into CPD-A2 (anticoagulant with increased glucose) and stored for 42 days either standing vertically or lying horizontally. It was reasoned that the horizontal position would facilitate

diffusion by increasing the plasma to cell surface ratio. They found that hemolysis was significantly greater and survival significantly lower in the concentrates stored in the vertical position than those stored in the horizontal position.

The significant differences in hemolysis may be due to the inability of substrates to diffuse from the overlying plasma into the deeper layers of the sedimented cells in the vertical position during the storage period. However, the percent of hemolysis in the units stored in the horizontal sedimented position was still unacceptably high (>1%).

7.4.2.2

Mixing Injury of Red Cells During Storage

Sedimentation can be prevented on Earth by continuous mixing. Dern et al. (2) studied the effect of intermittent mixing (5 times weekly), single mixing (once midway through storage) and non-mixing on the storage of CPD whole blood for 28 days. They demonstrated that intermittent mixing gave the best in vivo and in vitro results after storage, followed by single mixing and then non-mixing. Units with intermittent mixing showed an increase in ATP level and in post-infusion survival but a decrease in plasma hemoglobin when compared to the single- or non-mixing units.

However, injury caused by agitation (or mixing) to red cells was described as early as 1884 by Meltzer et al. (3). These authors observed that agitation of whole blood in glass tubes produced the release of hemoglobin from red cells and demonstrated that this was dependent on shear stress and on interactions between red cells and container wall surfaces.

Although the mechanism of the mixing lesion is poorly defined, it has been shown that mechanical mixing can result in loss of membrane lipid (4), reduction in the surface to volume ratio (5), increase in cation permeability (6) and decrease in deformability which correlates with poor post-infusion survival (7).

7.4.3.1

Platelet Storage on Earth: Mixing Injury

Storage of platelet concentrates under standard blood bank conditions requires continuous mixing. Work from several laboratories has shown that mixing of platelets during storage prevented extensive deterioration of platelet functions as measured by activation, yield and survival (8-11). For example, Murphy et al. (8) studied the effect of mixing on the survival of 48 hour stored

platelet concentrates. The mean survival ($T_{1/2}$) was 3.6 days for the mixed, 2.6 days for the non-mixed concentrates, and 4.2 days for fresh platelets. Morphology of platelets and the pH of the concentrate were also better maintained in the mixed units (9).

Several commercial mixing devices are currently available for storage of platelet concentrates. There are two general types, flatbed (horizontal) and rotary. Results from storage of platelet concentrates using two different five-day bags and four different mixing devices are summarized in Table I. As illustrated in Table I, the mode of agitation is extremely important for both in vivo and in vitro function of platelets. At the present time, the mechanism as to why only minor variations in the mode of mixing should have such significant effect on platelet storage has not been defined. It should be emphasized, however, that even with continuous mixing, the recovery, survival and function of stored platelets are decreased when compared to fresh platelets.

7.4.3.2

Contact Activation of Platelets

It is well established that platelet-platelet contact as well as platelet-container surface contact can cause alteration and activation of these cells. In other words, contact among platelets themselves and between platelets and foreign surfaces can induce platelet injury. Continuous mixing during storage facilitates contact activation of platelets. This may be one of the mechanisms which leads to decreased recovery, survival and function of stored platelets.

7.4.4

Leukocyte Storage on Earth

To date, it is necessary to transfuse leukocytes as soon as possible after collection (less than 6 hours) because these cells rapidly lose their integrity and function during storage. There are many unsolved problems with regard to leukocyte storage (18-21). For example, leukocyte collection under standard blood bank conditions invariably contains a large number of platelets and red cells. Interactions among these cells during sedimentation result in a fall in pH lack of diffusion of nutrients and clumping of leukocytes by platelet activation.

Little is known about the effect of mixing on leukocytes during storage. McCullough et al. (18) reported that mixing during storage, although it had no effect on the bacterial killing function of leukocytes, caused a

Table I. Effects of Methods of Agitation on in Vivo and in Vitro Characteristics of Platelets Stored for 5 Days at 22°C

| Method of Agitation | Polymers | | | | |
|---------------------|------------------|----------|------------------|-------|---------------------------------|
| | PL 732a | | CLX ^b | | |
| | Function | Survival | Recovery | Ref. | Function Survival Recovery Ref. |
| HORIZONTAL | | | | | |
| Flashed | +++ ^c | +++ | +++ | 12,13 | +++ 14-i6 |
| ROTARY | | | | | |
| Elliptical | ± | ± | ± | 13 | ± 14,15 |
| Tumbler | +++ | +++ | +++ | 13 | +++ 14-16 |
| Ferris Wheel | + | ± | ± | 12,13 | ± 17 |

^a polyolefin plastic; ^b PVC plasticized with TOTM;

^c +++: acceptable; ++: fair; +: equivocal; ±: unacceptable

reduction in chemotaxis (migration of leukocytes to a noxious stimulus such as bacteriae). The chemotactic response of leukocytes, an energy-dependent process, deteriorates first and to the greatest extent during storage when compared to other functions (19-21).

We have previously proposed that storage of blood at microgravity leads to marked energy conservation (see section 7.4.1.2.). Therefore, microgravity will better maintain energy-dependent functions of leukocytes, e.g. chemotaxis. Leukocyte function is maintained through a narrow pH range (7.0 to 7.5) (18). Storage at microgravity which prevents sedimentation would reduce the steep pH gradients to which leukocytes are subjected under standard blood bank conditions may in fact improve the viability of leukocytes.

7.5.1

Biomaterials and Blood Storage on Earth

A phthalate plasticizer is currently employed in the manufacture of blood bags. The evidence of the harmful consequence of the phthalate plasticizer has been reviewed in our original proposal (section 2.1.4, Biomaterials). Accordingly, industry has been engaged in extensive studies to develop alternative polymers.

In comparison to the standard PVC-DEHP blood bags, all alternative polymers developed to date have been deleterious to the red cell in that: 1) there is increased hemolysis; 2) the red cell potassium does not leak out to the same degree as the hemoglobin; 3) there is increased osmotic fragility; 4) the red cell survival at 21 days in CPD anticoagulant is reduced and 5) the red cell survival in all new polymer formulations utilizing CPD adenine anticoagulant is less than 70% (FDA Regulations require greater than 70% survival) after 35 days storage. These factors point to a selective membrane alteration. All attempts to define the mechanism of this alteration on earth have failed.

7.5.2

Biomaterials and Microgravity Blood Storage

The interaction of biomaterials and blood cells during storage is one of the multiple variables that can affect blood storage on earth (see above). Microgravity as provided by the space shuttle would eliminate the effect of two most important variables, i.e. sedimentation and the mechanical mixing injury to stored blood cells (see previous section), and is the only means by which the separation of the sedimentation, container and mechanical mixing lesions can be achieved. A comparison of the membrane changes of blood cells stored in different

polymer formulations at microgravity will give new insight into the membrane characteristics of blood cells upon storage.

In this project, we will utilize three FDA approved blood bags, one, the currently used PVC-DEHP bag, and two alternative polymer formulations, the PVC-TOTM and polyolefin bags. PVC-DEHP and PVC-TOTM contain the plastizers phthalate and trimellitate, respectively, whereas, polyolefin bags do not contain a plasticizer. The studies summarized below have been carried out in the Blood Bank Research Laboratory at Children's Hopspital (under the leadership of Drs. S. Kevy and M. Jacobson) as part of a cooperative program with industry for the in vitro and in vivo evaluation of alternative polymer formulations. Our results demonstrate the existence of a membrane abnormality due to the interaction of the polymer with the membrane of both the red cell and platelet as well as the affinity of the plasticizer for lipid.

7.5.3

Leaching of Plasticizer

The leaching of DEHP from PVC during blood storage is dependent on diffusion and is directly proportional to the lipoprotein concentration. Two aspects of diffusion are involved: 1) migration through the plastic to the internal surface of the container, and 2) diffusion of lipoprotein molecules toward and away from the container wall. Although only a fraction of the leached DEHP is associated with the red cell, this plasticizer has a membrane stabilizing effect (see below). In a previous study, we compared the degree of leaching of TOTM and DEHP and their effect on human fibroblast tissue culture. The leaching of TOTM by plasma is one-hundredth that of DEHP and is independent (unlike DEHP) of lipoprotein concentration. Furthermore, results from tissue culture studies showed that TOTM did not inhibit the growth of human fibroblasts, whereas DEHP was a potent inhibitor (22).

7.5.4

Lipid Association of Plasticizer

During storage there is an active exchange between the plasma and erythrocyte membrane lipids (23). It is thought that the neutral lipids other than cholesterol are not an integral part of the red cell membrane but represent small amounts of plasma lipoprotein material adsorbed to the surface of the membrane (24). Utilizing polyvinyl chloride plasticized with ¹⁴C-carbonyl labeled DEHP we were able to determine that the degree of leaching was limited by the lipoprotein concentration. Ultracentrifugation studies revealed that 93% of the

DEHP found in plasma was in the lipoprotein fraction. As shown in Table II, agarose gel chromatography demonstrated that the low density component bound more plasticizer than either the very low or high density components (25). There was little bound to albumin. The plasticizer is probably solubilized and transported in plasma in a manner similar to that of the triglycerides.

Table II. Association of ^{14}C -DEHP with Lipoprotein Components of Plasma

| DEHP Addition | % of Total Recovery | | | |
|---------------|---------------------|-----|-----|-----|
| | VLDL | LDL | HDL | Vi* |
| 23.3 mg/ml | 19 | 47 | 27 | 7 |

*VLDL: very low density lipoprotein; LDL: low density lipoprotein; HDL: high density lipoprotein; Vi: non-lipoprotein fraction

7.5.5

Membrane Stabilization Effect of DEHP

Red blood cells when exposed to DEHP are more resistant to hemolysis as compared to those cells which are stored in non-DEHP containers (26). The mechanism of the "protective" effect of DEHP on hemolysis is unknown. However, Seeman (27) demonstrated that there is a 50% inhibition of hemolysis when the membrane area of the intact erythrocyte expands by 2 to 3%. Possible mechanisms by which compounds could expand the membrane area are: 1) occupation of space within the membrane, 2) disordering and expansion of the lipid regions of the membrane, 3) induction of conformational changes in membrane proteins, and 4) adsorption to the membrane, thus altering the amount of water in contact with the membrane. Furthermore, Roth and Seeman (28) demonstrated that lipophilic anesthetics stabilize the red cell membrane against hypotonic hemolysis while drugs with low lipid solubility had no effect. It is conceivable, therefore, that DEHP, due to its lipophilic nature may stabilize the red cell membrane in a like manner.

7.5.6

Red Cell Studies

Storage of blood in alternative polymer containers (PVC-TOTM, the polyolefins, and etc.) resulted in increased hemolysis and osmotic fragility and decreased post-infusion survival as compared to PVC-DEHP bags. In

this section additional data from studies of hemolysis and osmotic fragility are included whereas the survival data have been reviewed in our original proposal.

7.5.6.1

Hemolysis Studies

The rate of hemolysis in stored blood is generally accepted as the primary initial screening procedure to indicate biocompatibility with the erythrocyte. In 1954, Gibson and Thorn (29) compared blood stored in glass and plasticized polyvinyl chloride containers, and demonstrated that the plasma hemoglobin level and the susceptibility to hemolysis in hypotonic 0.6% saline was evident at one week and markedly increased during the third week of storage in the "inert" glass container as compared to polyvinyl chloride formulations, whereas all other biochemical parameters measured were identical.

Paired studies were carried out in our laboratory to compare plasma hemoglobin, osmotic fragility, red cell electrolytes and glycolytic intermediates in blood stored in PVC-DEHP bags and alternative polymers. The average plasma hemoglobin level at one week was markedly different for PVC-DEHP and polyolefin but similar for the polyolefin and a polyolefin copolymer. By 3 weeks the plasma hemoglobin level for the polyolefin bag was 64.2 mg/dl with its PVC-DEHP control at 34.1 mg/dl; the polyolefin co-polymer had a level of 44.0 mg/dl with its PVC control at 27.5 mg/dl (means of 5 paired studied). Similar results were obtained with 5 other polyolefin derivatives. All exhibited significant differences by one week.

Studies were also performed comparing PVC-DEHP with an identical PVC formulation using a TOTM plasticizer. The average plasma hemoglobin for the PVC-DEHP controls was 33.4 mg/dl and the PVC-TOTM was 66.5 mg/dl. The rate of hemolysis of the PVC-DEHP and PVC-TOTM packs were quite different. This was evident within the first week of storage.

7.5.6.2

Osmotic Fragility Studies

Results from paired studies on osmotic fragility are shown in Figs. 1 and 2. We demonstrated that significant losses in stability as measured by hypotonic hemolysis occurred in red cells stored in containers fabricated with non-plasticized polymers and TOTM as compared to the PVC-DEHP containers (Figs. 1 and 2). Fragility curves were generated for each sample but only the percent hemolysis in 0.6% NaCl is shown. By the end of four weeks the percent hemolysis is 11 for PVC-DEHP

and 27.5 and 29.5, respectively for two of the experimental polymers (Fig. 1) . After 3 weeks of storage the PVC-DEHP units had 12.5% hemolysis and after four weeks 18.5% hemolysis whereas the PVC-TOTM units had 23.5 and 34.8 percent hemolysis at the corresponding storage times (Fig. 2). It should be emphasized that as early as one week after storage, significant differences were noted.

In the same paired studies, red cell electrolytes and glycolytic intermediate levels were identical for all polymers tested. All the aforementioned polymers have excellent gas transfer and two of them have been approved for 5 day platelet storage.

7.5.7

Platelet Studies

Therapeutic effectiveness of platelets is judged by three parameters: 1) Yield or Recovery: This is the term applied to the percent of the platelets that circulate following transfusion. 2) Survival: The duration in days that the platelets circulate. 3) Correction of the patient's bleeding time.

Previous studies from our laboratory has demonstrated that varying formulations of PVC bags plasticized with DEHP exert a profound effect on platelet yield and survival during storage (Table III). As shown in Table III, polymers #2 and #3 were suitable for storage up to 72 hours with normal survival whereas polymers #1 and #4 had markedly decreased survival within 48 hours. Both polymers 2 and 3 are currently used in blood banks for platelet storage up to 72 hours. It should be emphasized, however, that platelet recovery in the two latter polymers was only 40% of that of fresh platelets.

Table III. Effect of Four PVC-DEHP Formulations
on Platelet Recovery and Survival
(n=10)

| Plastic Pack | Storage Temp (°C) | Hrs. | Recovery (%) | Survival Days |
|--------------|-------------------|------|----------------|------------------|
| #1 | 22 | 0 | 51.2 \pm 1.5 | 8.6 |
| #2 | 22 | 0 | 61.3 \pm 2.2 | 8.5 |
| #3 | 22 | 0 | 58.6 \pm 2.7 | 8.3 |
| #4 | 22 | 0 | 42.5 \pm 3.1 | 7.9 |
| #1 | 22 | 48 | 43.5 \pm 2.9 | 2.9 ^a |
| #2 | 22 | 48 | 52.5 \pm 2.2 | 7.5 |
| #3 | 22 | 48 | 54.8 \pm 2.1 | 6.9 |
| #4 | 22 | 48 | 41.1 \pm 2.6 | 1.8 ^a |
| #2 | 22 | 72 | 35.0 \pm 1.6 | 7.2 |
| #3 | 22 | 72 | 35.2 \pm 1.4 | 7.1 |

^aThe 72-hour recovery and survival were not determined due to poor survival at 48 hours.

7.6

Summary

Clearly, from the above discussion storage of whole blood, platelet concentrates and leukocytes under earth's gravity, either in the sedimented state or with continuous mixing is not optimal. Storage of blood at microgravity in the space shuttle is expected to result in a greatly reduced perturbation of cell function. The continuous suspension of blood during storage in the shuttle at a microgravity environment will more closely mimic the relationship of the cells to the plasma in the circulatory system than does ground storage of blood. Continuous suspension of blood will 1) eliminate the accumulation of metabolites immediately surrounding blood cells, 2) increase the availability of substrates, 3) increase gas transport, 4) better maintain pH, and 5) reduce or eliminate the chances of contact activation for platelets.

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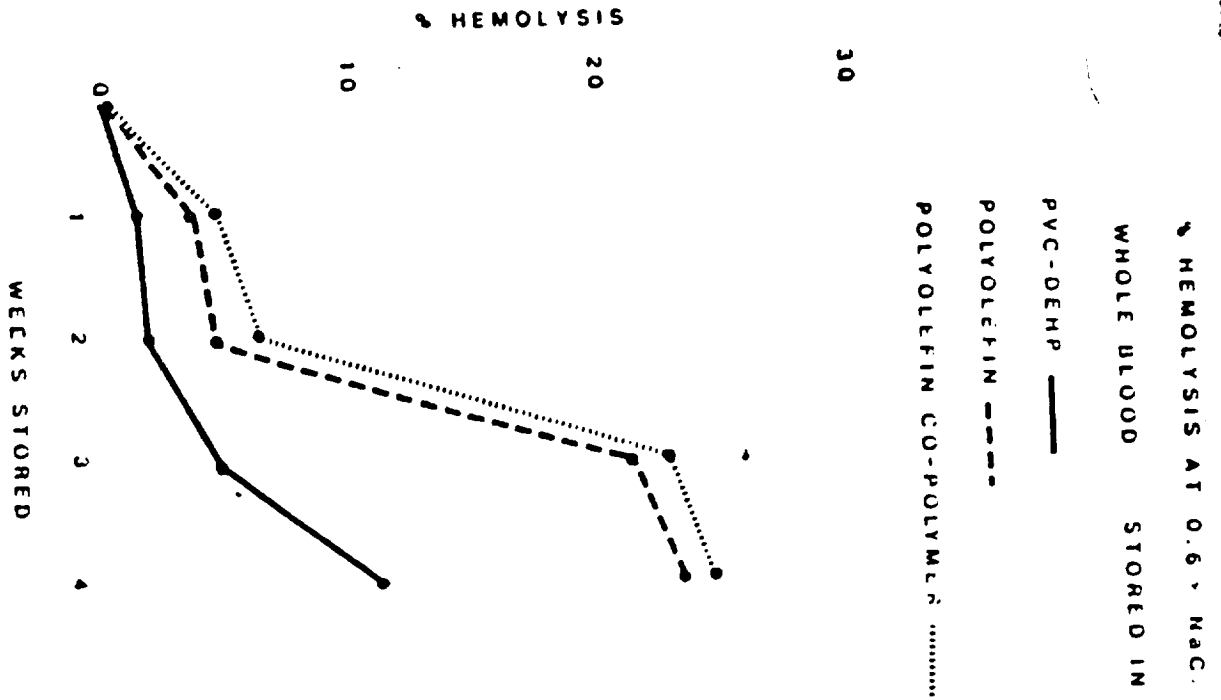


Figure 1

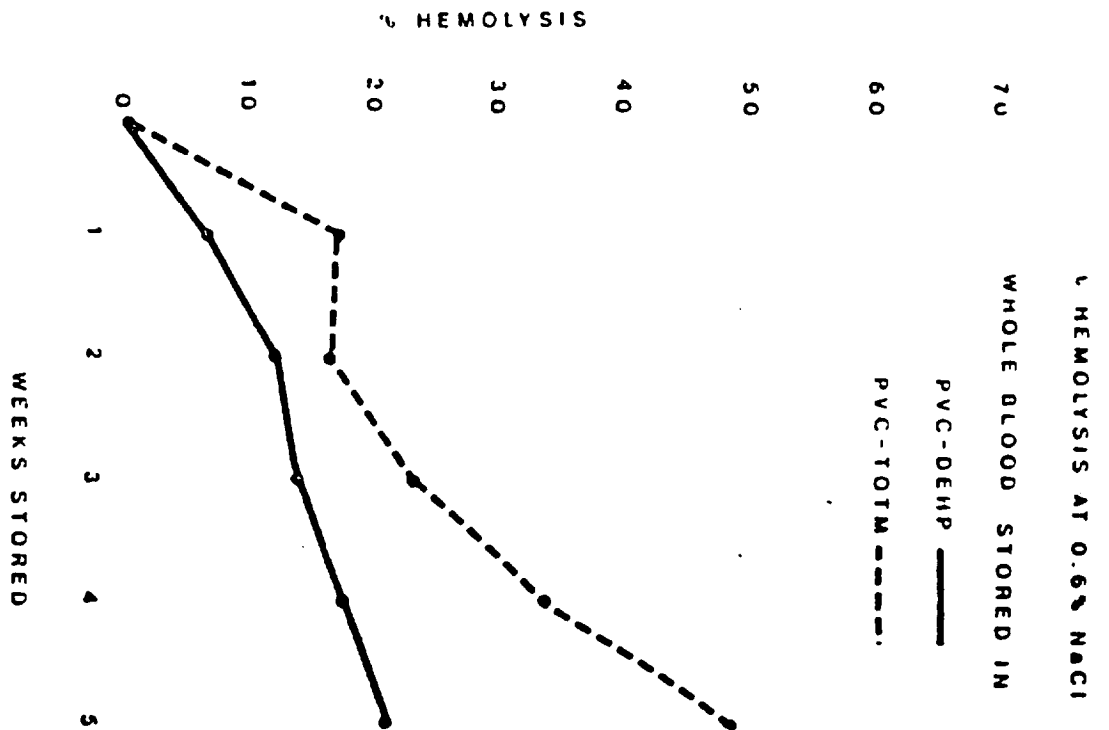


Figure 2

Osmotic fragility studies of whole blood stored in CPD A-1. Values shown are the mean of 5 experiments in which each donor unit was aliquoted into the indicated blood bag maintaining the standard surface/volume ratio.

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OF POOR QUALITY

Initial Blood Storage Experiment
(IBSE)

Scientific Experimental Protocol

Draft 5/85

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X

Initial Blood Storage Experiment

Experimental Protocol

1.0 INTRODUCTION

The Initial Blood Storage Experiment (IBSE) will involve pre and post flight biomedical operations. Blood will be collected from a group of blood donors, and sterile suspensions of blood cells will be separated into sealed plastic blood bags. The bags will be placed in temperature controlled environmental stainless steel dewars. The dewars will be housed in three modified middeck lockers. Two lockers comprise the flight payload; the third dewar and a functional IBSE unit will be held on the ground in the laboratory facility.

This experimental protocol details the procedures that will be performed at the onsite facility, Central Florida Blood Bank, and base laboratories. All procedures, where applicable, are in accordance with the American Association of Blood Banks Technical Standards, Eleventh Edition and the Good Manufacturing Practices for Blood and Blood Components of the Code of Federal Regulations.

2.0 BLOOD COLLECTION

2.1 Blood will be drawn from a group of 36 volunteer donors under standard operating procedures and aegis of the Central Florida Blood Bank (CFBB), which will be responsible for collection and preparation of the components used in the experiment. A member of the IBSE team will be present.

2.1.2 The donors will be preselected by CFBB according to the following guidelines:

- a.) having successfully donated blood previously for transfusion
- b.) having previously been shown to be negative for HBsAg
- c.) having previously been shown to be negative for irregular antibodies against red cell antigens
- d.) being of a single ABO and Rh type or at the discretion of the CFBB, two blood groups 12 units of one ABO and Rh for use as whole blood and 24 units of a second ABO and Rh for use as platelets and buffy coats.
- e.) having not ingested aspirin the day of blood collection or 5 days previous.
- f.) non-transfused males.
- g.) having previously been shown to be negative for anti-HTLV III antibodies, ie. suspected AIDS virus antibodies.

- 2.1.3 FDA approved standard CPD anticoagulant blood bags will be used.
- 2.1.4 CFBB criteria will govern the acceptance or rejection of all donors at the time of phlebotomy; as well as the disposition of any units found to be unsatisfactory for any reason.
- 2.1.5 Standard CFBB laboratory testing will be conducted by CFBB after the collection, from pilot samples obtained at the time of phlebotomy, i.e. ABO, Rh, antibody screening, syphilis testing, hepatitis testing and anti-HTLV III testing. All testing will be complete prior to pooling. All donor and donation records will be retained by CFBB.
- 2.1.6 Units collected as whole blood and not intended for component preparation will be stored at 1-6 C until the time of pooling and aliquoting.
- 2.1.7 Units intended for production of components will be stored at room temperature (20-24 C) for no more than six hours.

3.0 COMPONENT PREPARATION

- 3.1 All component preparation will be completed by the CFBB according to their standard operating procedures with the exception of the final plasma volume of platelet concentrates. These concentrates will be resuspended to volume of 65 ml.
- 3.2 The red cells and plasma from the units used for platelet and buffy coat preparation will be stored at 1-6 C for 21 days.

4.0 RECORDS

- 4.1 Pooling Records:
A Pooling Data Sheet is prepared prior to pooling each blood component. The accuracy of all data must be verified by a second person, who initials the sheet, before pooling. The following information must be documented:

- Component (whole blood, platelets, or leukocytes)
- Collection date
- Pooling date and time
- Temperature of pooling area (room temp, 5°C, etc.)
- Lot number and description of pooling bag
- Whole blood number of each component
- ABO and Rh type of each component
- Identity of person pooling
- Identity of person verifying records.

The time pooling is completed will be documented.

[illegible]

Initial Blood Storage Experiment
PRE-FLIGHT ALIQUOTING DATA SHEET

Whole Blood 250 ml

Date: _____

Polymer Code: PVC-DEHP = D
PVC-TOTM = T
Polyolefin = PO

Time Begun: _____

Time Completed: _____

Temperature of Aliquoting Area: _____

| Order | Bag ID Code# | Polymer | Gross Weight(g) | Net Weight(g) | Tech. | Review | Remarks |
|-------|-----------------|---------|--------------------|------------------|-------|--------|-----------|
| 1 | 1 | D | | | | | |
| 2 | 8 | T | | | | | |
| 3 | 15 | PO | | | | | |
| 4 | 2 | D | | | | | |
| 5 | 9 | T | | | | | |
| 6 | 16 | PO | | | | | |
| 7 | 3 | D | | | | | |
| 8 | 10 | T | | | | | |
| 9 | 17 | PO | | | | | |
| 10 | 4 | D | | | | | |
| 11 | 11 | T | | | | | |
| 12 | 18 | PO | | | | | Spare Bag |
| 13 | 5 | D | | | | | |
| 14 | 12 | T | | | | | Spare Bag |
| 15 | 19 | PO | | | | | |
| 16 | 6 | D | | | | | |
| 17 | 13 | T | | | | | |
| 18 | 20 | PO | | | | | |
| 19 | 7 | D | | | | | Spare Bag |
| 20 | 14 | T | | | | | |
| 21 | 21 | PO | | | | | |

Initial Blood Storage Experiment
PRE-FLIGHT ALIQUOTING DATA SHEET

Leukocytes 75 ml

Date: _____

Polymer Code: PVC-DEHP = D
PVC-TOTM = T
Polyolefin = PO

Time Begun: _____

Time Completed: _____

Temperature of Aliquoting Area: _____

| Order | Bag ID Code# | Polymer | Gross Weight(g) | Net Weight(g) | Tech. | Review | Remarks |
|-------|--------------|---------|-----------------|---------------|-------|--------|-----------|
| 1 | 22 | D | | | | | |
| 2 | 29 | T | | | | | |
| 3 | 36 | PO | | | | | |
| 4 | 23 | D | | | | | |
| 5 | 30 | T | | | | | Spare Bag |
| 6 | 37 | PO | | | | | |
| 7 | 24 | D | | | | | |
| 8 | 31 | T | | | | | |
| 9 | 38 | PO | | | | | |
| 10 | 25 | D | | | | | |
| 11 | 32 | T | | | | | |
| 12 | 39 | PO | | | | | |
| 13 | 26 | D | | | | | |
| 14 | 33 | T | | | | | |
| 15 | 40 | PO | | | | | |
| 16 | 27 | D | | | | | Spare Bag |
| 17 | 34 | T | | | | | |
| 18 | 41 | PO | | | | | |
| 19 | 28 | D | | | | | |
| 20 | 35 | T | | | | | |
| 21 | 42 | PO | | | | | Spare Bag |

Initial Blood Storage Experiment
PRE-FLIGHT ALIQUOTING DATA SHEET

Platelets 65 ml

Date: _____

Polymer Code: PVC-DEHP = D
PVC-TOTM = T
Polyolefin = PO

Time Begun: _____

Time Completed: _____

Temperature of Aliquoting Area: _____

| Order | Bag ID Code# | Polymer | Gross Weight(g) | Net Weight(g) | Tech. | Review | Remarks |
|-------|-----------------|---------|--------------------|------------------|-------|--------|-----------|
| 1 | 43 | D | | | | | |
| 2 | 50 | T | | | | | |
| 3 | 57 | PO | | | | | Spare Bag |
| 4 | 44 | D | | | | | |
| 5 | 51 | T | | | | | |
| 6 | 58 | PO | | | | | |
| 7 | 45 | D | | | | | |
| 8 | 52 | T | | | | | Spare Bag |
| 9 | 59 | PO | | | | | |
| 10 | 46 | D | | | | | |
| 11 | 53 | T | | | | | |
| 12 | 60 | PO | | | | | Spare Bag |
| 13 | 47 | D | | | | | |
| 14 | 54 | T | | | | | |
| 15 | 61 | PO | | | | | |
| 16 | 48 | D | | | | | |
| 17 | 55 | T | | | | | |
| 18 | 62 | PO | | | | | |
| 19 | 49 | D | | | | | Spare Bag |
| 20 | 56 | T | | | | | |
| 21 | 63 | PO | | | | | |
| 22 | 98 | T | | | | | |
| 23 | 99 | T | | | | | |

4.2 Records of Pre-flight Aliquoting of Pools:

A Pre-Flight Aliquoting Data Sheet is prepared prior to aliquoting each pool into the coded experiment blood bags. The order in which the bags are to be filled is pre-determined on the data sheet. The aliquoting technician will verify the labeled bags as being of the correct polymer and having the correct code. After the aliquoting, a second person will review the records and initial to verify accuracy.

5.0 POOLING

All pooling and aliquoting will be performed using aseptic techniques. All bench area will be washed down with a 10% bleach solution prior to use and after any blood spills.

5.1.0 Whole Blood Pool

12 units of whole blood will be pooled in groups of 6 units to make two pools. The two pools will then be mixed together to make one pool. Whole blood pooling may be carried out at room temperature, but after aliquoting the bags should be placed immediately at 1-6°C.

5.1.1 Remove the segments from each of the units of whole blood. Place the segments from each unit in separate plastic bags labeled with the corresponding whole blood number and store between 1-6°C for 21 days.

5.1.2 Using the Dupont Sterile Connection Device (SCD), connect the integral tubing from a unit of whole blood to the tubing of a cytoagglerator bag. Allow the whole blood to empty into the cytoagglerator bag. Once the bag is empty, clamp the tubing with a hemostat and heat seal the integral tubing in three places close to the port of the whole blood bag.

5.1.3 Disconnect tubing at the center seal. Repeat with 5 more units of whole blood.

5.1.4 Repeat the above procedure with the remaining 6 units of whole blood and a second cytoagglerator bag.

5.1.5 Once the second bag is filled, the two pooling bags must be mixed together. Using the SCD, connect the two cytoagglerator bags.

5.1.6 Hang one bag above the other. Allow the blood in the hanging bag to flow into the second bag, mixing gently until the hanging bag is empty. This should take about 10 minutes.

5.1.7 Reverse positions of the two bags and repeat until the two bags are mixed a total of ten times.

5.1.8 Store whole blood pool at 1-6°C until ready to aliquot.

5.2 Platelets

All platelet concentrates must rest for 1 hour and 15 minutes and be rotated for a minimum of 1 hour and 15 minutes prior to pooling. All platelet pooling will be performed at room temperature.

- 5.2.1 Pool 24 units of platelet concentrates into a 2 liter plasma transfer pack.
- 5.2.2 Using the SCD, connect a 2 liter plasma transfer pack with a unit of platelet concentrate. Hang the platelet unit above the transfer pack and allow the platelets to gravity feed into the transfer pack. When the platelet bag is empty, clamp the integral tubing with a hemostat. Heat seal the integral tubing 3 times close to the port of the platelet bag. Disconnect at the center seal.
- 5.2.3 Repeat until all 24 platelet units have been pooled.
- 5.2.4 Mix by inversion.
- 5.2.5 Store pooled platelets at room temperature until ready to aliquot.

5.3 Leukocyte Pool

Preparation of leukocytes will be performed under a laminar flow hood.

- 5.3.1 24 units of buffy coats will be sedimented with dextran to separate the leukocytes. Suspensions of leukocyte will be washed in plasma transfer packs. See white cell protocol, Section 14.3.
- 5.3.2 Connect a 2 liter plasma transfer bag to a unit of leukocytes, using the SCD. Hang the leukocytes above the plasma transfer pack and allow the leukocytes to gravity feed into the transfer pack. When the leukocyte bag is empty, clamp the integral tubing with a hemostat. Heat seal the integral tubing 3 times close to the port of the leukocyte bag. Disconnect the 2 bags at the center seal.
- 5.3.3 Repeat until all leukocytes have been pooled.
- 5.3.4 Mix by inversion.
- 5.3.5 Store pooled leukocytes at 1-6°C until ready to pool.

5.4 Plasma

- 5.4.1 Select 8 units of plasma from units used for platelet preparations for pooling into a 2 liter plasma transfer pack. The pooled plasma will be used for tests on platelets.
- 5.4.2 Using the SCD, connect a unit of plasma to a 2 liter plasma transfer pack. Hang the plasma above the transfer pack and allow the plasma to gravity feed into the transfer pack. When the plasma bag is empty, hemostat the integral tubing. Heat seal the tubing

close to the port of the plasma bag. Disconnect the 2 bags at the center seal.

5.4.3 Repeat until 8 bags of plasma have been pooled.

5.4.4 Mix by inversion.

5.4.5 Store pooled plasma at 1-6°C until needed.

6.0 ALIQUOTING

Each product pool will be aliquoted into 3 different, FDA OoB approved plastic formulations: PVC-DEHP, PVC-TOTM, and Polyolefin. Each whole blood and leukocyte pool will be aliquoted into 21 samples in 300 ml bags. The platelet pool will be aliquoted in 23 samples in 300 ml bags. There will be 7 bags of each plastic formulation for each whole blood and leukocyte pool. For the platelet pool, there will be 7 bags PVC-DEHP and 7 bags polyolefin and 9 bags PVC-TOTM. The total number of bags will be sixty-five. The volumes vary according to each product.

6.1 Prelabeling of bags

Each bag will be given a unique number which will identify the blood component, the type of plastic, the dewar and locker used for storage.

| | PVC-DEHP | PVC-TOTM | Polyolefin |
|-------------|----------|---------------|------------|
| Whole Blood | 1-7 | 8-14 | 15-21 |
| Leukocytes | 22-28 | 29-35 | 36-42 |
| Platelets | 43-49 | 50-56, 98, 99 | 57-63 |

Spare bags are: Whole Blood: 7, 12, 18

Leukocytes: 27, 30, 42

Platelets: 49, 52, 57

6.1.2 Code for location and storage condition

| ORBITER | | | | | | | EARTH | | | | | | |
|------------------|----|------|----|------|----|------|----------------------|----|------|----|------|----|------|
| | WB | LEUK | WB | LEUK | WB | LEUK | | WB | LEUK | WB | LEUK | WB | LEUK |
| A | 6 | 22 | 10 | 29 | 20 | 37 | D | 4 | 40 | 13 | 32 | 15 | 28 |
| B | 8 | 41 | 17 | 26 | 5 | 35 | E | 11 | 24 | 19 | 38 | 2 | 33 |
| C | 21 | 34 | 3 | 39 | 14 | 23 | F | 16 | 31 | 1 | 25 | 9 | 36 |
| P | 56 | 48 | | | | | P* | 60 | 50 | | | | |
| L | | | | | | | L | | | | | | |
| A | 62 | 53 | | | | | A | 47 | 98 | | | | |
| T | | | | | | | T | | | | | | |
| E | 43 | 58 | | | | | E | 51 | 63 | | | | |
| L | | | | | | | L | | | | | | |
| E | 54 | 99 | | | | | E | 59 | 46 | | | | |
| T | | | | | | | T | | | | | | |
| S | 44 | 61 | | | | | S | 45 | 55 | | | | |
| FRONT _____ BACK | | | | | | | FRONT _____ BACK | | | | | | |
| (TOP) | | | | | | | (TOP) _____ (BOTTOM) | | | | | | |

Letter codes are dewars. A and B are cold dewars stored in one orbiter locker. C is a cold dewar and PLATELETS is a warm dewar stored in the orbiter locker. D and E are cold dewars stored in one earth locker. F is a cold dewar and PLATELETS* is a warm dewar stored in the other earth locker.

6.2 Whole Blood

6.2.1 Whole blood will be aliquoted in 250 ml volumes in the following prelabeled 300 ml bags and stored at 1-6°C until loaded in the dewars:

1. PVC DEHP 7 bags
2. Polyolefin 7 bags
3. PVC TOTM 7 bags

6.3 Platelets

6.3.1 Platelets will be aliquoted in 65 ml volumes in the following prelabeled 300 ml bags and stored at room temperature (20-24°C) until loaded in dewar:

1. PVC DEHP 7 bags
2. Polyolefin 7 bags
3. PVC TOTM 9 bags

6.4 Leukocytes

6.4.1 Leukocytes will be aliquoted in 75 ml volumes in the following prelabeled 300 ml bags and stored at 1-6°C until loaded in the dewars:

1. PVC DEHP 7 bags
2. PVC TOTM 7 bags
3. Polyolefin 7 bags

6.5 Procedure for aliquoting

6.5.1 Whole blood

- 6.5.1.2 Mix the whole blood pool well prior to aliquoting and again between each bag.
- 6.5.1.3 Place a 300 ml bag to be filled on scale and tare for the weight of the bag.
- 6.5.1.4 Using the SCD, connect one cytoagglomerator bag containing whole blood pool with the 300 ml bag for aliquoting.
- 6.5.1.5 Allow 250 g of whole blood to flow into the above prelabeled bag.
- 6.5.1.6 When bag is filled with the appropriate volume, clamp with a hemostat. Heat seal tubing 3 times close to the port of the aliquoted bag. Disconnect the 2 bags at the center seal.
- 6.5.1.7 Repeat with each prelabeled bag for whole blood until 11 bags are filled or cytoagglomerator bag is empty.
- 6.5.1.8 Using the sterile docking device connect second cytoagglomerator bag to a bag for aliquoting. Repeat steps 6.5.1.2-6.5.1.7.
- 6.5.1.9 The blood remaining in the cytoagglomerator bag will be aliquoted into plastic tubes for use in baseline studies. See 7.0.
- 6.5.1.10 Store whole blood aliquots at 1-6°C until ready to load in the dewar.

6.5.2 Platelets

- 6.5.2.1 Mix platelet pool well before aliquoting and between each bag during aliquoting.
- 6.5.2.2 Place bag for aliquot on scale and tare for the weight of the bag.
- 6.5.2.3 Using the SCD, connect the platelet pool with a prelabeled bag for aliquoting.

6.5.2.4 Allow 65 g of platelet suspension to flow into aliquoting bag. Clamp with hemostats when the appropriate volume has been aliquoted. Heat seal the integral tubing 3 times, close to the port of the aliquot bag. Disconnect bags at the center seal.

6.5.2.5 Repeat until all 23 bags are filled.

6.5.2.6 The platelet suspension remaining in the pooled bag will be aliquoted into tubes for baseline studies. See 7.0.

6.5.2.7 Aliquoted platelets will be stored at room temperature (20-24°C) until loaded in the dewar.

6.5.3 Leukocytes

6.5.3.1 Mix leukocyte pool well before aliquoting and between each bag during aliquoting.

6.5.3.2 Place bag for aliquot on scale and tare for the weight of the bag.

6.5.3.3 Using the SCD, connect the leukocyte pool with a prelabeled bag for aliquoting.

6.5.3.4 Allow 75 g of leukocytes to flow into aliquoting bag. Clamp with hemostats when the appropriate volume has been aliquoted. Heat seal the integral tubing 3 times, close to the port of the aliquot bag. Disconnect bags at the center seal.

6.5.3.5 Repeat until all 21 bags are filled.

6.5.3.6 The leukocyte suspension remaining in the pooled bag will be aliquoted into tubes for baseline studies. See 7.0.

6.5.3.7 Aliquoted leukocytes will be stored at 1-6 until loaded in the dewar.

7.0 SAMPLES FOR BASELINE STUDIES

Time zero samples are those taken at the time of aliquoting. Their purpose is to verify the condition of the products pre flight. All assays outlined in the protocol will be performed on time zero samples. Each investigator will receive samples according to the description below.

7.1 Whole blood

Whole blood pool must be mixed well prior to aliquoting.

7.1.1 Using a hemostat, clamp the integral tubing of the cytoagglerator bag. Cut the end of the tubing. Place the tubing in a prelabeled

centrifuge tube. Release the hemostat and allow the require volume (listed in 7.1.2) to flow into the tube. When the tube is filled with the require volume reclamp the hemostat and repeat until all whole blood tubes are filled.

- 7.1.2 Whole blood will be aliquoted into the following tubes labeled whole blood and place on ice or to maintain a temperature of 1-6°C.

| # of Tubes | Tube Volume | Volume Needed | Investigator |
|------------|-------------|---------------|--------------------------------|
| 3 | 50 ml | 50 ml | M. Jacobson (A.B.[1]) |
| 1 | 50 ml | 30 ml | I. Szymanski |
| 2 | 15 ml | 10 ml | M. Jacobson D. Ausprunk |
| 2 | 10 ml | 10 ml | M. Jacobson (A.S.) |
| 2 | 15 ml | 5 ml | M. Jacobson (S.K.) W. Curby |

7.2 Platelets

Platelets must be well mixed prior to any aliquoting.

- 7.2.1 Using a hemostat, clamp the integral tubing of the platelet bag. Cut the end of the tubing. Place the tubing in a prelabeled centrifuge tube. Release the hemostat and allow the require volume (listed in 7.2.2) to flow into the tube. When the tube is filled with the require volume reclamp the hemostat and repeat until all platelet tubes are filled.

- 7.2.2 Platelets will be aliquoted into the following tubes labeled platelets and left at room temperature:

| # of Tubes | Tube Volume | Volume Needed | Investigator |
|------------|--------------|---------------|--------------|
| 1 | 3 ml syringe | 1.5 ml | M. Jacobson |
| 1 | 50 ml | 30 ml | F. Chao |
| 1 | 50 ml | 15 ml | F. Chao |
| 1 | 15 ml | 5 ml | I. Szymanski |
| 1 | 15 ml | 4 ml | D. Ausprunk |
| 1 | 15 ml | 3 ml | M. Jacobson |
| 1 | 15 ml | 1 ml | W. Curby |

7.3 Leukocytes

Leukocyte pool must be well mixed prior to aliquoting.

- 7.3.1 Using a hemostat, clamp the integral tubing of the leukocyte bag. Cut the end of the tubing. Place the tubing in a prelabeled centrifuge tube. Release the hemostat and allow the require volume

(listed in 7.3.2) to flow into the tube. When the tube is filled with the require volume reclamp the hemostat and repeat until all whole blood tubes are filled.

- 7.3.2 Leukocytes will be aliquoted into the following tubes labeled leukocytes and place on ice or to maintained at a temperature between 1-6°C:

| # of Tubes | Tube Volume | Volume Needed | Investigator |
|------------|-------------|---------------|--------------|
| 2 | 50 ml | 30 ml | F. Lionetti |
| 2 | 15 ml | 5 ml | D. Ausprunk |
| 1 | 2 ml | 1 ml | W. Curby |

8.0 PACKAGING AND SHIPMENT OF BLOOD

- 8.1 Whole blood, red blood cells, leukocyte preparations and plasma will be packed and shipped on wet ice to maintain at a temperature of 1-6°C.
- 8.2 Platelets will be packed in IBSE shipping racks and shipped at 20-24°C.
- 8.3 A member of the IBSE team will observe the packing of blood components prior to shipment. This person will accompany the blood in the helicopter to KSC. A second person will accompany the ground stored blood to Boston.

9.0 STORAGE OF ALIQUOTED POOLED SAMPLES

Of the sixty-three aliquoted bags, fifty-six will be used in the experiment. One extra bag of each plastic and each component will be available in the event a replacement is required. There are 2 dewars to each locker and 4 lockers total. 2 lockers will remain in the laboratory and 2 lockers will be placed on the orbiter. The following table is a the description of how the products and the specific bag types will be divided.

Earth stored aliquots

Whole blood:

3 PVC-DEHP
3 PVC-TOTM
3 Polyolefin

Leukocytes:

3 PVC-DEHP
3 PVC-TOTM
3 Polyolefin

Orbiter stored aliquots

3 PVC-DEHP
3 PVC-TOTM
3 Polyolefin

3 PVC-DEHP
3 PVC-TOTM
3 Polyolefin

Platelets:

| | |
|--------------|--------------|
| 3 PVC-DEHP | 3 PVC-DEHP |
| 4 PVC-TOTM | 4 PVC-TOTM |
| 3 Polyolefin | 3 Polyolefin |

A total of 56 bags, 18 whole blood, 18 leukocytes and 20 platelets.

9.1 Whole blood and leukocytes

Whole blood and leukocytes will be stored in a total of 6 cold dewars. Three of the cold dewars will be left in the laboratory under controlled conditions and three will be placed on the orbiter. Each cold dewar will contain 3 whole blood aliquots and three leukocyte aliquots in the configuration described in 6.1.2.

9.2 Platelets

Platelets will be stored in a total of 2 warm dewars (22°C). One warm dewar will be left in the laboratory under controlled conditions and one will be placed on the orbiter. Each dewar will contain 9 platelet aliquots and one "dummy" bag in the configuration described in 6.1.2.

The "dummy" bag will contain platelets from the pool and aliquoted into a PVC-TOTM bag.

10.0 LOADING OF DEWARS

- 10.1 This activity requires 3 IBSE staff members (2 CBR and 1 ADL staff) to manipulate the hardware. These three individuals will work in a restricted area, O + C Building, room 2249 or 2251, until all dewars are loaded and ADL closeout of the lockers is complete. All clerical entries will be verified and initialed by a second person.
- 10.2 Two Curby cranes for suspending IBSE hardware over the lockers will be in place for the loading procedure.
- 10.3 Each dewar will be prelabeled by ADL (A-D for Orbiter, E-H for Earth-stored dewar). The bag types, blood components, and temperature of each dewar by label are defined in section 6.2.
- 10.4 The microgravity-stored dewars will be loaded at KSC and the ground stored will be loaded simultaneously in Boston. The procedure is identical for microgravity and Earth-stored dewars.
- 10.5 Selection of blood components:

- 10.5.1 Prior to loading the dewars, filled, coded bags will be separated into 6 identical groups for whole blood and leukocytes, each containing 3 whole blood units (one in each type of plastic bag), 3 leukocyte units (one in each type of plastic bag), and 2 identical groups for platelet units.

- 10.5.2 Before loading commences, the code number of each bag to go in the dewar will be recorded on the appropriate worksheet.
- 10.5.3 A plastic protective drape will be placed over the lower locker assembly to prevent spillage into the hardware in the unlikely event of a broken blood bag during the loading procedure.
- 10.6 Loading will begin with platelet bags since they are stored at room temperature.
 - 10.6.1 Platelet bags will be secured in vertical ___ mm holders as described in section ___, arranged by bag type as designated in 6.1.1. The final position of each coded bag will be recorded on the appropriate worksheet.
 - 10.6.2 The platelet holders will be joined by rubber bands maintaining the relative positions defined in section 6.1, and the entire unit slipped into the $22 \pm 1^{\circ}\text{C}$ dewar compartment.
- 10.7 Cold-stored components intended for a single dewar (3 whole blood and 3 leukocytes) will be removed from refrigerated storage and brought to the dewar loading site. Each bag will be removed from its protective plastic overwrap and wiped dry with towelling to remove condensation prior to loading.
 - 10.7.1 Whole blood and leukocytes will be loaded alternately in the arrangement described in section 6.1.2, beginning at the bottom of the $5 \pm 1^{\circ}\text{C}$ dewar.
 - 10.7.2 Each bag must be folded at the top, label side up, maintaining maximum surface area against the air flow grid surface. The top of the bag is folded back onto the label and is positioned at the top of the individual compartment.
 - 10.7.3 The folded blood bag is placed horizontally in a parallel folded Teflon sheet which facilitates placement of the blood bag into the hardware without sliding the bag across any rough surfaces. Then, while the blood bag is held in place from the opposite side, the teflon sheet "envelope" is withdrawn. Corners of the plastic bag may not protrude out of the prescribed area in order for the hardware to lower properly into the locker.
 - 10.7.4 To fully contain any protruding bag corners and to ensure ease of lowering, each loaded bag will be encircled in the hardware by Kapton tape (Connecticut Hard Rubber). Whole blood will be encircled by 1" tape and leukocytes will be encircled by 1/2" tape. Precut strips of tape will be attached to a vertical metal bar in the hardware and circled in the direction of the 3 adjacent bars and finally around the open space allowed for loading.
- 10.8 The 2-dewar unit is ready for ADL closeout. Repeat with Earth-stored lockers and dewars.

11.0 SUMMARY OF PRE-FLIGHT TIMELINE

The launch is scheduled for 7 a.m., _____ 1985. The IBSE lockers must be turned over to NASA 12 hours prior to that, 7 p.m., _____ 1985. In order to minimize pre-flight exposure to 1 x g, the donor blood will be collected early on the morning before and all subsequent preparations are defined within that 12-13 hour period.

11.1 Day Prior to Launch

6:00 - 9:00 a.m. Collection of blood from CFBB donors.
6:00 - 8:00 24 donors for platelet and leukocyte production.
8:00 - 9:00 12 donors for whole blood.

8:00 - 10:30 Platelet concentrates rest and rotate.

8:00 - 11:00 Leukocytes prepared from buffy coats at CFBB.

9:00 - 2:00 p.m. Whole blood units pooled and divided into coded IBSE bags, at CFBB, 1 person.

10:30 - 2:00 Platelets and leukocytes pooled and divided into coded IBSE bags, at CFBB, 2 people.

2:00 - 3:30 Prepared Blood components and 1 IBSE staff transported to KSC by helicopter. Other IBSE personnel needed for loading the dewars at KSC must travel by car.

3:30 - 7:00 Load orbiter and ground dewars.
ADL closeout of orbiter and ground lockers.

** 7:00 p.m. Turn over orbiter dewars to NASA.

12.0 ADL FLIGHT PREPARATIONS

The experiment requires eight nearly identical environmental chambers. During the preflight preparatory phase, suspensions of human blood formed elements will be prepared, introduced and sealed into plastic bags (containers), which will then be mounted in fixed positions within the environmental chambers as described above. These chambers will then be closed, the associated environmental control systems (temperature control and air supply) will be activated and the entire system will be enclosed in modified NASA storage lockers for flight. The environmental chambers must then operate continuously from the time of insertion and closure of the blood preparation into the chambers until the locker assembly has been removed from the orbiter and returned to IBSE personnel.

12.1 The design of the chambers and their associated environmental control systems must assure that the following environmental specifications will be met from the point of preflight loading and closure to the termina-

tion of flight and the lockers have been turned over to the IBSE personnel (see 12.1 Table I).

12.1 TABLE I

| Chambers needed | Whole Blood | Platelets | Leukocytes |
|--|--------------|---------------|--------------|
| Capacity | 250ml | 65ml | 75ml |
| Thickness of bags with blood | 3.0cm | 0.5cm | 1.5cm |
| Temperature Range | 5° \pm 1°C | 22° \pm 1°C | 5° \pm 1°C |
| Air | Cabin air | Cabin air | Cabin air |
| Orientation of the bag during take off | Horizontal | Vertical | Horizontal |

The air flow through the chambers will be a minimum of 85 cc/min.

12.2 All blood preparations will be placed in 300 ml volume plastic bags. Three different formulations of plastics will be used for the bags. The dimension of the filled blood bag will vary with the component place in it due to differing volumes being inserted into the bags. The configuration of the chambers must ensure that the bags do not come in contact with one another, are held in position to avoid any shifting, and are spaced in uniform distances. The chambers must provide a constant flow of air to maintain the exchange of oxygen and carbon dioxide across the wall of the blood bags containing the formed elements of the blood.

12.3 The power and air supply must have capability of maintaining the restrictions stated in the Table I for a total of 10 days. The temperature must remain within stated ranges. Should there be an interruption of power, the temperature within the chambers containing red cells and leukocytes should not rise above TBD for more than TBD at any one time. The temperature of the chamber containing platelets should not rise at above TBD for more than TBD at any one time.

12.4 In addition to the design of the environmental chambers, ADL is responsible for the following:

12.5 Proper placement of the insert holding the blood bags in the dewars, activation of the environmental system and closure of the lockers.

12.6 Packaging and delivery of the lockers to KSC personnel.

12.7 Assuring that the proper environmental conditions are maintained pre and post flight.

12.8 Placement of the lockers on and off the orbiter

The lockers will be loaded and delivered to KSC personnel a minimum of 12 hours prior to launch. During this period, specified environmental conditions must be maintained within the lockers.

13.0 POSTFLIGHT ALIQUOTING OF SAMPLES FOR TESTING

13.1 Labeling of plastic tubes for aliquoted samples

All blood will be aliquoted into prelabeled prearranged plastic centrifuge tubes, test tubes or syringes. All labeling of tubes will be completed prior to the landing of the orbiter. All labeling and aliquoting will be performed in a secluded area by authorized personnel.

13.2 Whole Blood

For each of the 18 whole blood units label with corresponding code numbers the following tubes:

| # of Tubes | Tube Volume | Volume Needed | Investigator |
|------------|-------------|---------------|-----------------------|
| 3 | 50 ml | 50 ml | M. Jacobson (A.B.[1]) |
| 1 | 50 ml | 30 ml | I. Szymanski |
| 2 | 15 ml | 10 ml | M. Jacobson |
| | | | D. Ausprunk |
| 2 | 10 ml | 10 ml | M. Jacobson (A.S.) |
| 1 | 15 ml | 5 ml | M. Jacobson (S.K.) |
| 1 | 15 ml | 5 ml | W. Curby |

13.3 Leukocytes

For each of the 18 leukocyte units label with corresponding code numbers the following tubes:

| # of Tubes | Tube Volume | Volume Needed | Investigator |
|------------|-------------|---------------|--------------|
| 2 | 50 ml | 30 ml | F. Lionetti |
| 1 | 15 ml | 5 ml | D. Ausprunk |
| 1 | 15 ml | 5 ml | W. Curby |

13.4 Platelets

For each of the 20 platelet units label with corresponding code numbers the following tubes:

| # of Tubes | Tube Volume | Volume Needed | Investigator |
|------------|--------------|---------------|--------------|
| 1 | 3 ml syringe | 1.5 ml | M. Jacobson |
| 1 | 50 ml | 30 ml | F. Chao |
| 1 | 50 ml | 15 ml | F. Chao |
| 1 | 15 ml | 5 ml | I. Szymanski |
| 1 | 15 ml | 4 ml | D. Ausprunk |
| 1 | 15 ml | 3 ml | M. Jacobson |
| 1 | 15 ml | 1 ml | W. Curby |

13.5 Removal of bags from the dewars

TBD

13.6 ADL will unload the lockers (separate procedure TBD).

13.7 CBR personnel will unload the dewars (separate procedure TBD).

The platelet dewar will be unloaded and platelets place on a rotator for approximately 30 minutes prior to aliquoting.

13.8 Aliquoting

13.8.1 For each component a specified volume will be aliquoted into pre labeled tubes according to the charts in 13.2-13.4

13.8.2 Using a coupler with a spike on each end, enter a bag. Be sure the roller clamp is closed. Hang the bag above the rack of pre-labeled tubes. Open the clamp and allow the required volume of sample to flow into premarked tubes.

13.9 Once the earth stored dewars are unloaded and samples aliquoted the same procedure will be repeated for the orbiter stored dewars.

13.10 Aliquoted samples will be stored at the following temperatures until received by the investigator:

| | |
|----------------------------|---------|
| Whole blood and leukocytes | 1-6°C |
| Platelets | 20-24°C |

13.11 Only when all aliquoting operations are complete, will each investigator will receive a rack of coded samples. All assays will be performed as described in 14.0 Detailed Experimental Procedures

14.0 DETAILED EXPERIMENTAL PROCEDURES

14.1 Erythrocyte Studies (Technical Proposal 3.2.2)

Sample Aliquoting and Designated Responsibility for the Erythrocyte StudyPersonnel:

D. Ausprunk (D.A.); A. Byrnes (A.B.); W. Curby (W.C.); M. Jacobson (M.J.); S. Kevy (S.K.); A. Scanlan (A.S.); I. Szymanski (I.S.); L. Wolfe (L.W.).

| TESTS | Whole Blood Volume (ml) | Responsible Person | Performance Site |
|---|----------------------------|-----------------------|---------------------|
| <u>Physical Measurements</u> | | | |
| Mean Critical Hemol. vol | 5 | W.C. | on site |
| Osmotic Fragility | 5 | S.K., A.S. | on site |
| Morphology and Shape Change (E.M.) | 10 | D.A. | on site and Boston |
| pH, PO ₂ , PCO ₂ | | A.S. | on site |
| <u>Membrane Properties</u> | | | |
| Intact Cells - Lipids | 10 | M.J. | on site and Boston |
| Ghosts | 10 | A.B., L.W. | on site |
| Spectrin Extraction | 50 | A.B. | on site and Boston |
| Spectrin-Actin-4.1 Binding | | A.B. | Boston |
| Changes in Membrane Proteins | | A.B. | Boston |
| Membrane Glycoproteins | 50 | M.J. | on site and Boston |
| <u>Metabolites</u> | | | |
| ATP, DPG, Glucose | 10 (red top tube) | A.S. | on site and Boston |
| <u>Electrolytes</u> | | | |
| WB K ⁺ , Na ⁺ , plasma K ⁺ , Na ⁺ | 10 (autoclear tube) | A.S. | on site and Boston |
| plasma Hgb, WB, Hgb | | A.S. | on site and Boston |
| <u>Plasma</u> | 50 | M.J. | on site |
| <u>Lipids</u> | | M.J. | on site and Boston |
| plasticizer quant. | | M.J. | on site and Boston |
| coagulation factors | | M.J. | on site and Boston |
| membrane dust | | M.J., A.B. | |
| <u>Immunology</u> | 30 | I.S. | on site and Boston |

ON-SITE STUDIES

18 Samples

A. MEMBRANE PROPERTIES

1. Intact Cells

a) 10 cc of each whole blood sample will be washed by centrifugation 3X with cold buffered saline and the buffy coat removed by passing through a cellulose column. The cells will be resuspended in 3 cc of PBS.

b) 0.5 cc will be saved for electronic Hematology parameters by Coulter analysis.

(1) Lipid Analysis: 1 ml cells will be extracted with chloroform-isopropanol and back extracted with 0.05 M KCl. The extracts will be stored frozen (-20°C) and will be transported back to the laboratory at CHMC in Boston for completion of analysis (see below for methodology).

2. Red Cell Ghosts

a) 50 cc of whole blood will be prepared into ghosts by a modification of the method of Dodge (see below). The ghosts will be counted electronically by Coulter analysis.

(1) Protein Structure will be analyzed by two-dimensional SDS gel electrophoresis. Ghosts will be solubilized in 0.5% SDS plus 0.5 mM EDTA and with and without 0.02 M dithiothreitol, incubated 1 hr (37°C), frozen and brought back to Boston (-40°C). The ghosts will be analyzed in Boston by 5% polyacrylamide gel electrophoresis, as described by Fairbanks et al. The resulting gels will be stained for protein with Coomassie Blue and for glycoproteins with PAS (see below).

(2) Spectrin Extraction: Spectrin dimer will be isolated from 10 cc of ghosts by low ionic strength extraction at 37°C and purified by gel chromatography at 4-6°C (see below). Samples will be stored at 4-6°C and transported back to CHMC laboratory in Boston. See protocol below for Spectrin-Actin Protein-4.1 assay.

(3) Glycoprotein Labeling: See section under platelets.

B. RED CELL METABOLISM

1. 10 ml of whole blood will be used to determine the metabolic state of the red cell.

a) 2,3 Diphosphoglyceric Acid: 1 ml of whole blood will be immediately deproteinized by pipetting into 3.0 ml Trichloroacetic Acid in a 15 ml polypropylene centrifuge tube.

(1) Vortex immediately and sit in ice for 5 min. Centrifuge at 2500 x g for 10 min and take off clear supernatant. Freeze (-20°C) and transport back to CHMC laboratory in Boston for completion. See protocol.

b) ATP: Adenosine-5-triphosphate

(1) Pipet into a 15 ml centrifuge tube 4.0 ml of 0.6 N perchloric acid (ice cold) and 1.0 ml whole blood. Mix well (Vortex).

(2) Allow to stand in ice for 10 min then centrifuge for 10 min at 2500 x g at 4°C.

(3) Take off supernatant and freeze at -40°C for transport back to CHMC lab in Boston for completion. See protocol.

c) Glucose

(1) Pipet 0.4 ml whole blood into 15 ml centrifuge tube into 3.6 ml 3% trichloroacetic acid. Centrifuge for 10 min at 2500 x g. Take off supernatant and freeze for transportation back to CHMC lab in Boston for completion.

d) Plasma Hemoglobin

(1) 3 ml of whole blood will be placed into a plastic 7 ml centrifuge tube and centrifuged in a Sorvall at 4°C for 30' at 5000 rpm. The supernatant is taken off and respun. The supernatant will be stored at 4-6°C for transport back to the CHMC lab in Boston. See protocol.

e) The remaining whole blood will be transported at 4-6°C back to the CHMC lab for K⁺, HgB, Na⁺ determinations.

2. Plasma: 25 ml of whole blood in a 50 ml plastic centrifuge tube will be centrifuged at 4200 x g for 5 min. The plasma will be separated.

a) 3 ml aliquot will be frozen at -20°C for factor analysis and transported back to the CHMC laboratory.

b) 3 ml aliquot will be extracted for lipid analysis in chloroform-methanol and back extracted with 0.2 M KCl. Extracts will be stored frozen (-20°C) for return to the CHMC laboratory. See protocol for analysis.

c) The remaining aliquot will be frozen at -20°C and transported back to the CHMC lab for plasticizer quantitation. See protocol for analysis.

C. Physical Properties

1. Osmotic Fragility: 1 ml of whole blood will be used for this test. A unopette osmotic fragility kit will be used for these determinations. Fragilities will be determined with and without incubation for 1 hour at 22°C in buffered saline. See protocol for analysis.

2. pH: 0.5 ml of whole blood will be placed into a 1 ml plastic vial and stored frozen at -20°C until pH is read.

PREPARATION OF RBC GHOSTS

Modification of method of Dodge, J.T., Mitchell, C., and Hanahan, D.J.:
Arch. Biochem. Biophys. 100:119-130 (1963).

Media

5mM Isotonic phosphate buffered saline, pH 8.0.
0.1 mM EGTA

Procedure

1. Approximately 4 cc anticoagulated blood washed 3x with cold buffered saline. Resuspended in ~3 cc of B.S. Send ~.5 cc or less to lab for Hgb and RBC. WBC must be removed when cells hemolyzed.
2. 2.5 cc of resuspended RBC's diluted to 35 cc in a graduated cylinder with cold 30 mosm buffer and inverted several times to ensure complete mixing and hemolysis of the red cells. (Ghosts should be kept at ~4°C at all times during the procedure). Approximately 9 cc apportioned to each of 4 centrifuge tubes. Graduate rinsed once with ~5 cc 30 mosm buffer, which is added to tubes.
3. Centrifuge tubes balanced, centrifuged at 9000 x g for 10 min in cold.
4. Hemolysate aspirated and button of ghosts resuspended with stream of cold 30 mosm buffer from a wash bottle.
5. Ghosts in each centrifuge tube are resuspended with ~10 ml of 30 mosm buffer. Tubes are inverted several times to ensure complete suspension.
6. Centrifugation, aspiration of supernatant and reusspension is repeated, using cold 30 mosm buffer, until supernatant is completely clear and ghost button is white. At this point all ghosts are combined in one centrifuge tube, resuspended in 10 cc, 30 mosm buffer and collected a final time (usually a total of 4-5 centrifugations will be required).
7. If a small button of debris and/or unhemolyzed BRC's remains below the ghost button, this is left behind when ghosts are combined in the last step (6 above).
8. Ghosts are resuspended at the desired volume (usually 5 ml) with 30 mosm media. They may be washed and stoerd in other media as desired.
Recovery = usually 90-95%
Yield = 5 ml of ghosts with a concentration of ~ 3×10^9 ghosts/ml.

Ghosts may be counted in Coulter counter or in RBC counting chamber (phase microscopy) after standing 30 min.

SDS GEL ELECTROPHORESIS

REFERENCE: Fairbanks, G., Steck, T.L., and Wallach, D.F.H. Electrophoretic analysis of the major polypeptides of the human erythrocyte membrane. *Biochemistry* 10:2606-2617 (1971).

as modified by -- Steck, T.L. Cross-linking of the major proteins of the isolated erythrocyte membrane. *J. Mol. Biol.* 66:295-305 (1972).

as modified by -- Laemmli, U.K. Cleavage of structural problems during the assembly of the head of bacteriophage T4. *Nature* 227:680 (1970).

STOCK SOLUTIONS:

1. Conc A+B: Acrylamide (20 g) (USE GLOVES)
Bis (0.75 g)
Water to 100 ml. Freeze in 150 ml lots (-20°C)
2. Gel Buffer, pH 7.4 (x10):
Tris 24.25 g Final conc = 0.4 M
NaOH 4.0 g " " = 0.2 M as NaAc
Na₂EDTA 3.7 g " " = 0.02 M
HAc to pH 7.4
Water to 500 ml. Freeze in 10 ml lots.
3. Solubilizing Solution (x5):
Tris 1.21 g Final conc = 0.1 M
Na₂EDTA 0.37 g " " = 0.01 M
Sucrose 50.0 g " " = 50%
Titrate to pH 7.4 with 1M HCl
Water to 100 ml. Freeze in 5 ml lots.
4. 20% SDS: SDS 100 g
Water to 500 ml. Freeze in 100 ml lots.
5. 1.5% Ammonium Persulfate:
Amm. Per. 0.15 g
Water to 10 ml.
(USE GLASS TO PIPET) (make up fresh)
6. 0.5 TEMED: TEMED 0.05 ml
Water to 10 ml. Freeze in 5 ml lots. .
(USE GLASS TO PIPET) (make up fresh)
7. Coomassie Blue Stain:
MeOH 454 ml
H₂O 454 ml
Glacial Acetic Acid 92 ml
Coomassie Brilliant Blue 1.25 g
Filter with Whatman No. 1 filter paper.

8. Fixative: MeOH 500 ml
 H₂O 450 ml
 HAc 50 cc

9. Destain: HAc 10%

B. Making Gel

1. Set up plates (use medium spacers and 20 well comb).
2. Make the following solution:

| <u>Stock solution</u> | <u>Amount</u> |
|-----------------------|---------------|
| A + B | 12.0 cc |
| X10 | 6.0 cc |
| 20% SDS | .6 cc |
| Water | 32.4 cc |
| 1.5% AP | 6.0 cc |
| .05% TEMED | 3.0 cc |

3. Pour solution between plates, place comb, and wait for polymerization.
4. Remove comb and place gel into chamber.
5. Fill chamber top and bottom with electrophoresis buffer.

X10 200 ml
10% SDS 20 ml
Water to 2 l

6. Place 30 ug of protein in each well

a. Solubilizing Solution

X5 Sol. soln. = .8 ml
20 % SDS = .4 ml
Dithiothreitol = ~ 25 mg
Pyronin Y = dash

b. To solubilize protein add:

33 ul Sol. soln. to 100 ul of sample
Boil 3 minutes.

7. Run gel at ~ 75 volts until tracking dye reaches the bottom of the gels (takes about 2 hrs).
8. Put gel in fixative overnight shaking.
9. Stain with Coomassie Blue, shaking, 1 hr.
10. Destain with 10% HAc, shaking.

ATP: ADENOSINE -5'-TRIPHOSPHATE ASSAY

REF: Method Bucher, Th., Biochim.
Biophys. Acta (Amst) 1, 129,
(1947 a Denneman, H.A., ges.
exp. Med. 134, 335) (1961).

REAGENTS:

Solutions I:

1. 0.151 gms Glycerat-3-phosphate (BM#127116) dissolved in 100 ml of Triethanolamine buffer (Sigma #665-5).

Solution stable for four months at 4°C.

Solution II:

2. 0.018 gms -Nicotinamid-adenin-dinucleotide NADH (BM#127345) in 10 ml of distilled water.

Solution stable for four weeks at 4°C.

3. a) Glycerin-3Phosphat-dehydrogenase/Triosephosphat Isomerase (BM#127787).
b) 3-Phosphoglycerat-Kinase/Glycerinaldehyd-3-phosphate-Dehydrogenase (BM#108456).
4. 0.6N Perchloric Acid:
86.1 ml of 70% Perchloric Acid diluted to 1000ml with distilled water in a volumetric flask.
5. ATP standard (Sigma #A-3127) 100 um/ml
.0623 gms/ml of distilled water
Aliquots stored in freezer for daily use.
Dilute 25 ul of standard in 2.5 ml distilled water just prior to use.

SPECIMEN:

Since ATP is rapidly decomposed, specimen should be deproteinized as soon as possible.

DEPROTEINIZATION:

1. Pipet into a 15 ml centrifuge tube:
4.0ml 0.6N Perchloric Acid (ice cold)
1.0 ml blood and mix well

2. Allow to stand at room temperature for 10 min. then centrifuge for 10 min at approximately 3000 rpm (Sorvall). Use supernatant for assay. Store clear supernatant at -80°C.

SPECTROPHOTOMETRIC MEASUREMENTS:

Wavelength: 340 nm
Glass cuvette: 1 cm light path
Temperature: 20-25°C

PROCEDURE:

Pipette into cuvette:

2.0 ml Solution I
0.2 ml Solution II
0.2 ml supernatant fluid, unknowns, diluted standards, etc.

Mix by inverting with parafilm cover. Read optical density E_1 .
Add 10 μ l of BM suspension #127787 and 10 μ l of BM suspension #108456.

Mix by inverting with parafilm cover, wait until reaction stops (approx. 10 min) and read optical density E_2 .

$$E_1 - E_2 = E$$

CALCULATIONS:

$$239 \times E_{340 \text{ nm}} = \text{ATP}/100 \text{ ml}$$

$$\frac{\text{mg ATP}/100 \text{ ml}}{507 \text{ (MW of ATP)}} \text{ -- gms\% of hgb} = \text{uMoles ATP/gm hgb.}$$

:

2,3 - DIPHOSPHOCYLCLERIC ACID ASSAY

REF: Sigma Technical Bulletin
No. 35-UV

REAGENTS:

- A. -DPHN, Prewighted Vial, Stock No. 340-101
Each vial contains 1.0 mg -DPNH
Stable for over one year at room temperature while kept dark and
dessicated.
- B. Triethanolamine Buffer Solution, Stock No. 665-5
Triethanolamine Buffer containing Magnesium ions and EDTA.
Stable 6 months or longer when stored at 0-5°C.
- C. Adenosine - 5' - Triphosphate (ATP)
Vial containing 500 mg of Product No. A-5394
Sigma Grade ATP, Disodium Salt
Store below 0°C. Stabel one year or longer.
c'. Reconstitute vial with 5 ml Triethanolamine Buffer, Stock No.
655-5 (reagent B). Stable several months when stored frozen.
- D. GAPD/PGK Enzymes, Stock No. 366-2
Glyceraldhye Phosphate Dehydrogenase and Phosphoglyceric Phosphokinase
suspension in Ammonium Sulfate.
Mix by inverting before each use (DO NOT SHAKE).
Stable at least 6 months when stored at 0-5°C.
- E. Phosphoglycerate Mutase (PGM), Stock No. 665-3
Phosphoglycerate Mutase Enzyme suspension in Ammonium Sulfate.
Mix by inverting before each use (DO NOT SHAKE).
Stable at least 6 months when stored at 0-5°C.
- F. Phosphoglycolic Acid, Stock No. 665-2
50 mg preweighted vial. Stable when stored below 0°C.
f'. Reconstitute vial with 5.0 ml water. Stable for several
months when stored frozen.
- G. Trichloroacetic Acid 8%, Stock No. 665-8
Approximately 8% Trichloroacetic Acid (w/v).
Stable for at least 6 months when stored at 0-5°C.
- H. DPG Standard (BM#105821) 1 um/ml
0.0083 gms/10 ml of distilled water
0.0083 gms/100 ml of distilled water
Aliquots stored in freezer for daily use.

2,3 DIPHOSPHOCYLIC ACID ASSAY

SPECIMEN:

2,3-DPG appears to be stable in whole blood for at least 2 hours when stored in an ice bath. Trichloroacetic filtrates are stable up to 2 weeks when stored at 0-5°C.

DEPROTEINIZATION:

Pipett 1.0 ml of freshly drawn blood (heparin, ACD or CPD) into 3.0 ml of cold 8% Trichloroacetic Acid, Stock No. 655-8.

Shake vigorously for several seconds. Keep the mixture cold for an additional 5 minutes to assure complete protein precipitation.

Centrifuge 5 to 10 minutes at approximately 3000 rpm (Sorvall) to obtain a clear supernatant.

SPECTROPHOTOMETRIC MEASUREMENTS:

Wavelength: 340 mμ
Glass cuvette: 1 cm light path
Temperature: 20-25°C

PROCEDURE:

1. Into a 1 mg DPNH vial, Stock No. 340-101 pipette:
8.0 ml Triethanolamine Buffer Solution (Stock No. 665-5).
Cap and invert several times to dissolve the DPNH.
Stable up to one week when stored at 0-5°C. (DO NOT FREEZE).
2. Into two cuvettes marked BLANK and TEST, pipette the following:
(NOTE: ONLY ONE BLANK IS NEEDED FOR EACH SERIES OF TESTS)

| | <u>BLANK</u> | <u>TEST</u> |
|--|--------------|-------------|
| Mixture from Step 1 | 2.5 ml | 2.5 ml |
| ATP Solution (Reagent C) | 0.1 ml | 0.1 ml |
| Water | 0.25 ml | |
| Protein Free Filtrate | --- | 0.25 ml |
| (From Step B under Preparation of Protein Free Filtrate) | | |

Mix by inversion and add:

| | | |
|------------------------|---------|---------|
| GAPD/PGK Enzymes | | |
| (Reagent D) | 0.02 ml | 0.02 ml |
| PGM Enzyme (Reagent E) | 0.02 ml | 0.02 ml |

Mix by inversion
Wait approximately 5 minutes

3. Read and record OD₃₄₀ mu of both the BLANK and TEST. Cuvette using water as reference.
4. To each cuvette add: 0.1 ml phosphoglycolic acid (Reagent F). Mix by inversion. Let stand for 25-30 minutes (to allow reaction to go to completion).
5. Read and record OD₃₄₀ mu of BLANK and TEST using water as reference.

CALCULATIONS:

- a) OD BLANK = OD of BLANK (step 3) - OD of BLANK (step 5).
- b) OD TEST = OD of TEST (step 3) - OD of TEST (step 5).
- c) Calculate OD by subtracting a) from b);
$$OD = (OD\ TEST) - (OD\ BLANK)$$
- d)
$$\begin{array}{lcl} \text{uMoles of 2,3-DPG} & = & \frac{(OD) \times (3.0)}{(6.22) \times (0.0625)} = (OD) \times (7.7) \\ \text{per ml of blood} & & \end{array}$$

The factors are explained as follows:

- a) 3.0 represents the volume of liquid in the cuvette.
- b) 6.22 is the millimolar extinction coefficient for -DPNH at 340 mu.
- c) 0.0625 represents the volume of original sample in ml in the reaction mixture.

NOTE

You can also use above answer to calculate 2,3-DPG levels on the basis of Packed Cells, or Hemoglobin as follows:

$$\begin{array}{lcl} 1) \text{ uMoles of 2,3-DPG} & & (\text{uMoles of 2,3-DPG} \\ \text{per ml Packed Cells} & = & \text{per ml Whole Blood} \times (100) \\ & & \hline & & \text{Hematocrit in percent} \end{array}$$

Ex: If 2,3-DPG level is 2.2 uMoles per ml Whole blood and Hematocrit is 45%, then:

$$\begin{array}{lcl} \text{uMoles of 2,3-DPG} & = & (2.2) (100) \\ \text{per ml of Cells} & & \hline & & = 4.9 \end{array}$$

$$\begin{array}{l} 2) \text{ uMoles of 2,3-DPG} \\ \text{per grams Hemoglobin} = \end{array} \frac{\begin{array}{l} (\text{uMoles of 2,3-DPG} \\ \text{per ml Whole Blood} \times (100)) \\ \hline \text{grams Hemoglobin per 100 ml} \end{array}}$$

Ex: If 2,3-DPG level is 2.2 uMoles per ml Whole
blood and Hemoglobin level is 15 grams, then:

$$\begin{array}{l} \text{uMoles of 2,3-DPG} = \\ \text{per grams Hemoglobin} \end{array} \frac{(2.2) (100)}{(15)} = 14.7$$

Tetramethylbenzidine (TMB) Method for
Measuring Supernatant Hemoglobin

Reagents:

1. TMB Reagent, 1% (Aldrich Chemical Co.)

Dissolve 1 gram of TMB in 90 ml of glacial acetic acid and dilute up to 100 ml in a volumetric flask with distilled water. The reagent's reaction with hemoglobin will remain stable for at least 5 weeks despite some darkening of color if stored at 4°C.

2. Hydrogen Peroxide, 1% (30%, Fisher #H-325)

Dilute 3.3 ml of 30% hydrogen peroxide to 100 ml in a volumetric flask with distilled water. Prepare daily.

3. Acid Diluent Solution, 10% Glacial Acetic Acid

A 10% by volume acid diluent solution is prepared by adding 100 ml of glacial acetic acid to 900 ml distilled water. Stability of reagent is at least one month at R.T.

4. Platelet-Poor Hemoglobin "Free" Plasma (PPHFP), Diluent for standards and samples

Prepare from fresh citrated whole blood clarified by centrifugation and frozen in aliquots at -20°C. Prior to use, thaw and centrifuge. In order to compensate for the hemoglobin in this plasma diluent, a plasma blank is included with every run. The absorbance reading of this blank is then subtracted from the standards and the diluted samples.

Preparation of Hemoglobin Standards:

1. Packed red cells (hematocrit 70-80%) prepared from a unit of citrated whole blood should be lysed with distilled water (3 ml H₂O per ml red cells). Stroma and other debris should be removed by centrifugation and the hemoglobin concentration of the lysate should be determined by the cyanmethemoglobin method (Protocol #9). Aliquots should be frozen at -20°C.

2. Prepare a 200 mgs % standard from lysed cells:

Example:

W.B. Hgb of lysed cells = 11.1 gms%

$$\frac{11,000}{200} = \frac{10}{x}$$

or .181 10 ml distilled water
1.81 100 ml distilled water = 200 mg % solution

3. Prepare a working standard of 100 mg% with equal amounts of PPHFP and the 200 mg% standard solution.

| <u>Conc. of Standard</u> | <u>Amt. of 100 mg% Soln.</u> |
|--------------------------|------------------------------|
| 5 mg% | 1 ul |
| 25 mg% | 5 ul |
| 50 mg% | 10 ul |
| 75 mg% | 15 ul |
| 100 mg% | 20 ul |

Plot O.D. versus concentration.

Preparation of Samples for Assay:

Supernatant or plasma samples will be prepared by filling a sufficient number of 12 mm polypropylene tubes to provide enough plasma or supernatant for assay. The amount of blood required will, of course, be dependent upon the hematocrit of the blood.

Centrifuge the tubes in the Sorval at 5000 RPM for 20' at a temperature of 10°C.

Pipet the supernatant/plasma into another 12 mm polypropylene tube using a plastic pipette. Clear any entrained red cells from the sample by repeating the centrifugation, and pipetting the supernatant/plasma into a separate glass tube.

Spectrophotometric Measurements:

Instrument : Unicam spectrophotometer
Wavelength : 515 nm
Glass cuvette : 1 cm light path
Temperature : 20-25°C

Assay Procedure:

1. For each unknown, blank, plasma bank, and standards, measure 1 ml of 1% TMB into a test tube and add 20 ul of plasma or standard to each tube.
2. Add 1 ml of 1% H₂O₂ solution to each tube. Mix and let stand at room temperature for 20 minutes.
3. Following incubation add 10 ml of 10% CH₃COOH, mix well and wait 10 minutes before measuring absorbancy at 515 nm.
4. Calculate concentration of hemoglobin from standard curve.

pH Determination

Instrument:

Radiometer pH meter 4
Micro Electrode Unit
Circulation Thermostat

Operation:

1. Be sure the electrode water jacket is full of circulating water and the thermometer is set for the desired temperature.
2. Remove the glass electrode off its holder and press the suction cap against the plastic tip, with your thumb and suck distilled water through the capillary electrode until a few drops have passed the suction cap.
3. Fill the polyethylene tip and the capillary electrode with the buffer solution (7.381 pH).
4. Stop the suction and lift the polyethylene capillary out of the buffer solution.
5. Put the glass electrode back in its holder with the polyethylene tip immersed in the KCl pool of the calomet electrode (this is the measuring position).
6. Set the pH meter at READING.
7. Rotate the BUFFER ADJUSTMENT knob of the pH meter until the meter reads the pH of the buffer solution.
8. Set the pH meter to STAND BY.
9. Flush the electrode with distilled water, by sucking it through the capillary.
10. Suck air through the electrode for 3-5 seconds to dry its capillary.
11. Place the glass electrode in its resting position but with the polyethylene tip outside the beaker.
12. Mix the blood and insert the polyethylene tip into the tube or segment.
13. Press the suction cap against the plastic tip and suck blood into the capillary electrode until one drop is visible in the suction cap. Air bubbles must not be present in the polyethylene tip and in the glass capillary electrode.

14. Place the glass electrode in its measuring position, and read the pH when the meter has come to rest. Take down the result (the actual pH).
15. Immerse the polyethylene tip in a buffer solution for rinsing purposes. (Buffer solution is used for the rinsing because it prevents poisoning of the capillary electrode. The pH of the rinsing buffer should be between 7.3 and 7.4). Now create the suction and the rinsing buffer will clear the capillary electrode of the blood.
16. Rinse the glass capillary electrode with distilled water, refill with buffer of pH 7.38 and place the electrode in its resting position.

DETERMINATION OF RED CELL HEMOGLOBIN CONTENT

Instrument: Coleman Jr. Spectrophotometer or Unicam Spectrophotometer

The standard curve is set up by increasing dilutions of the Hycel Cyanmethemoglobin Standard. For the 5.0 ml volume employed in the unknown samples the undiluted standard corresponds to 20.0 Gm/dl hemoglobin. Dilutions are made to correspond to 15 Gm/dl, 10 Gm/dl, 5 Gm/dl and zero. Dilutions must be made with the Hycel Cyanmethemoglobin Reagent -- never with water.

Place five test tubes in a rack. Mark the tubes 20, 15, 10, 5 and 0. Dilutions must correspond to the following table:

| | | | | | |
|--------------------|--------|--------|--------|--------|--------|
| Gm/dl Hemoglobin | 20 | 15 | 10 | 5 | Blank |
| Volume of Standard | 6.0 ml | 4.5 ml | 3.0 ml | 1.5 ml | none |
| Volume of Reagent | none | 1.5 ml | 3.0 ml | 4.5 ml | 6.0 ml |

Transfer the dilutions to well matched cuvettes. Set the instrument to the proper wave length, 540 mμ or filter. Adjust the instrument so the Blank tube has zero Optical Density or 100% Transmission. Take the readings for the standard and plot Optical Density on straight graph paper and % Transmission on semi-log graph paper.

Determining Unknown Sample:

Place 5.0 ml of Reagent in 19 x 150 mm cuvette test tube. Add exactly 0.02 ml of blood and mix contents. Read against Reagent Blank. Transfer reading to standard curve and obtain hemoglobin concentration in Gm/dl.

THE COLORIMETRIC DETERMINATION OF GLUCOSE

REAGENTS:

- A. O. Toluidine Reagent, Stock No. 635-6
Store in dark at room temperature.
Caution: Do not pipet by mouth. Avoid contact with skin.
Do not inhale fumes.
- B. Trichloroacetic Acid Solution, 3% (W/V), Stock No. 635-3
Store in refrigerator at 0-5°C.
- C. Glucose Standard Solution, Stock No. 635-100
Standardized at 1.0 mg/ml (100 mg/100 ml or 5.5 m mol/l) with
Benzoic Acid added as preservative
Store in refrigerator at 0-5°C.
- D. Hyland Q-Pak--Chemistry Control Serum 1
List No. 045-030
Store between 2° and 8°C before and after reconstitution.

SPECIMEN:

Glucose can be determined on whole blood, plasma, or serum. At room temperature, glucose in blood undergoes glycolysis at a rate of approximately 5% per hour.

DEPROTEINIZATION:

Into a centrifuge tube, pipet:

0.4 ml sample

3.6 ml of 3% Trichloroacetic Acid, Stock No. 635-3

Mix well by shaking. Allow to stand approximately 5 minutes to precipitate proteins.

Centrifuge (5-10 minutes) until clear supernatant is obtained in the RC-3 at 3000 rpm. Store clear supernatant at 0-5°C, if determination is to be within a few hours. Otherwise freeze clear supernatant.

INSTRUMENT:

Practically any photoelectric colorimeter that transmits light in the range of 620-650 nm can be used.

Instrument: Coleman Jr.

Wavelength: 635 nm

Cuvette: 19 x 150 mm

NOTE: Instrument readings should be made in terms of Absorbance (A) for use in calculations and for preparation of calibration curves.

PROCEDURE WITH DEPROTEINIZATION

For whole blood and markedly icteric or hemolyzed samples.

CAUTION: Avoid inhaling fumes resulting from the O-Toluidine Reagent. It is suggested that the procedure be carried out in a well ventilated area or hood. DO NOT pipet this reagent by mouth.

1. Label test tubes or cuvetts BLANK, STANDARD, CONTROL, TEST 1, TEST 2, etc.

| <u>TO BLANK ADD:</u> | <u>TO STANDARD ADD:</u> | <u>TO CONTROL & TESTS ADD:</u> |
|------------------------|-------------------------|------------------------------------|
| 0.1 ml water | 0.1 ml Glucose Standard | 1.0 ml supernatant |
| 0.9 ml 3% TCA Solution | Solution | |
| Stock No. 635-3 | Stock No. 635-100 | |
| | 0.9 ml 3% TCA Solution | |
| | Stock No. 635-3 | |

2. To each tube add:
CAUTION: DO NOT PIPET BY MOUTH
5.0 ml O-Toluidine Reagent, Stock No. 635-6
Mix by lateral shaking.
3. Place all tubes in a vigorously boiling water bath for exactly 10 minutes.
NOTE: If results are calculated from a standard that is assayed with the Test, the boiling period can be 10 ± 1 minute.
4. Quickly remove all tubes and cool to room temperature by placing in tap water for approximately 3 minutes.
5. Transfer contents of tubes to cuvetts and read Absorbance of STANDARD and Tests at 635 ± 15 nm, using BLANK as reference. Complete readings within 30 minutes.

CALCULATIONS:

Use of Standard:

$$\text{Glucose (mg/100 ml)} = \frac{^A\text{Test}}{^A\text{Standard}} \times 1000^*$$

*Represents the concentration of the Glucose Standard (mg/100 ml).

NOTE: If the reading of Test indicates a Glucose concentration greater than 250 mg/100 ml, dilute the test with an equal volume of O-Toluidine Reagent, Stock No. 635-6. Read Absorbance of the diluted Test and multiply result by 2.

FLAME PHOTOMETRY

SODIUM AND POTASSIUM DETERMINATIONS

REF: Instrumentation
Laboratory Manual 143

EQUIPMENT:

IL Model 143 Flame Photometer
Compressed Air - Supply with a minimum pressure of 25#/in²
site gauge showing clean oil and with a flow of
moisture-free air of 0.5 ft³/min.
Fuel: Propane, (instrument grade)
Dade Dilutor Model 200

REAGENTS: (Instrumentation Laboratory, Inc.)

#35000 Stock Lithium Concentrate 1500 mEq Li/liter
dilute 20 ml stock to 2,000 ml with distilled water
#33203 150 mEq K/l
#35050 50 mEq Na/liter/100 mEq K/liter
#35100 100 mEq Na/liter/5 mEq K/liter
#97517-50 140 mEq/Na/liter/2 mEq K/liter
#97518-50 120 mEq/Na/liter/2 mEq K/liter
#97519-50 160 mEq/Na/liter/8 mEq K/liter

OPERATION:

1. Turn the front panel toggle switch to the ON position.
This allows ignition to take place.
 - a. Open the propane Shut-off valve located at the top of the fuel cylinder, one-half counter clockwise turn.
 - b. Open laboratory air manifold fully.
2. Turn ON/OFF switch to its upper position. The Flame On indicator light should come on and the faint sparkling sound cease within a matter of a few seconds.
3. Place a lithium blank solution or distilled water on the sample stand, raise the stand and aspirate for at least five minute before calibrating.

SAMPLE AND STANDARD PREPARATION:

1. Lithium Diluent - It is imperative that the lithium concentration in all calibrating standards and unknown samples be kept constant. Dilute (20 ml) #3500 Stock Lithium Concentrate 1500 mEq Li/liter to 2,000 ml with distilled water.

2. Calibrating Standards - Once the lithium diluent is prepared, dilute working standard can be made. Dilute all calibrating standards and also all biological unknown TWO HUNDRED TIMES with the lithium diluent. This is done via the Dade Dilutor with 50 ul of sample and 9.95 ml of diluent.
NOTE: It is recommended that approximately one ounce disposable plastic cups be used to hold standards and samples for analysis.

ANALYSIS OF DILUTED SAMPLES:

1. As recommended aspirate lithium diluent through the atomizer for several minutes before calibration. Doing this allows the diluent to thoroughly circulate around and wet the walls of the atomizing chamber, thus preventing the collection of droplets on the atomizer walls and instability in the readout.
2. Change the lithium solution and aspirate a fresh lithium diluent.
3. Set the needle on the Lithium Response Meter at the center of the reference triangle with the Lithium Set Control.
4. Set both the Sodium and Potassium Digital Concentration Displays to 0000 with their respective Zero Controls.
5. Aspirate an appropriate working standard 140 Na/5 K and switch the Potassium Range Selector Switch to the required position (20 for Serum).
6. Set both the Sodium and Potassium Digital Concentration Displays to the corresponding values of this standard with the respective Balance Controls. For example, the 140 Na/5 K working standard would be calibrated at 1400 and 0500, respectively.
7. Replace the standard with an unknown sample correctly diluted with lithium diluent and aspirate. Read and record the concentration of the unknown from the Sodium and Potassium Digital Concentration Display.
NOTE: Initially, it is advisable to recheck the calibration of the instrument after every five to ten unknowns. After ten to fifteen minutes of use, the calibration, typically, need be check less frequently.

INSTRUMENT SHUT DOWN:

1. After completing your determinations, aspirate lithium diluent through the unit for several minutes. This serves to thoroughly wash the atomizer and burner system.
2. Turn the propane Shut-Off Valve at the top of the fuel cylinder, clockwise (closed). Do not overtighten this valve, finger tight is adequate.
3. Close the air manifold fully.
4. Place the ON/OFF Switch in its lower position.

MAKING GHOSTS IN RC 2B OR 5B SS34 ROTOR
(All done at 4°C)

1. Solutions for making ghosts:

a. 0.15 M NaPO₄ Stock
titrate .015 M Na₂HPO₄ 7H₂O with .15 M NaH₂PO₄ H₂O until pH = 7.4

b. PBS
50 ml 0.15 M NaPO₄ stock
8.77 g NaCl

c. SP8
33.3 ml of 0.15 M PO₄
can also make it 0.5 mM EGTA = 2.5 cc of 200 mM stock
1 l = total volume
pH to 8 with NaOH

2. Spin down whole blood (go up to 12 K rpm, then cut out rotor).
3. Remove plasma.
4. Suspend and mix RBC's with PBS, spin down RBC (go up to 12 K rpm, then cut out rotor), aspirate off supernatant.
5. Repeat #4 2 more times.
6. Suspend and mix cells with SP8, spin down ghosts at 20 K rpm for 10-15 min. Aspirate supernatant and any white cells at the bottom of the tube.
7. Repeat #5 until ghosts are pure white (4-8 times depending on the amount of blood in the tube).

MAKING SPECTRIN EXTRACT

Unless otherwise indicated, done at 4°C in SS34 rotor in RC2B or RC5B centrifuge.

1. Make ghosts.
2. Suspend and mix ghosts in ice cold 0.1 mM PO_4 (20 cc of 5P8/1).
3. Spin at 20 K rpm = 50 kg for 20 min, aspirate off supernatant.
4. Repeat #2 and 3.
5. Incubate ghosts, shaking at 37°C for .5 hr.
6. Place ghosts into 60 TI rotor and spin for 1 hr at 39 K rpm = 110 kg in Sorvall or Beckman ultracentrifuge.
7. Aspirate and save supernatant = Spectrin extract.
8. Read OD^{280} against H_2O = Spectrin concentrate.

MAKING SPECTRIN DIMER (All done at 4°C)

1. Make spectrin from 50 cc of whole blood.
2. Concentrate spectrin to about 5 mg/ml.
3. Make extract 10% sucrose.
4. Load extract on 12 x 1000 mm column
(column gel = Biorad A-15).

A-15 Buffer
150 mM NaCl
10 mM Tris
.1 mM EDTA
.02% Na Azide

5. Run at 3 cc/hr, collect 20 min fractions.
6. Chromatograph.

SPECTRIN ACTIN 4.1 BINDING ASSAY

A. Solutions

Actin Buffer = Buffer A

| | |
|--------------------------|----------------------------|
| 2 mM Tris-HCl pH 8 | 2 ml of 1 M stock |
| 0.2 mM ATP | ~0.111 g (variable M.W.) |
| 0.5 mM DTT | 77 mg |
| 0.2 mM CaCl ₂ | 0.2 ml of 1 M stock |
| | <u>total vol = 1 liter</u> |

High Salt Buffer = HS Buffer

| | |
|------------|--------------------|
| 1 M NaCl | 5.84 g |
| 59 M Tris | 0.7139 g |
| (pH 7.4) | |
| 2.7 mM DTT | 0.0416 g |
| 0.9 mM ATP | <u>0.536 g</u> |
| | total vol = 100 ml |

[store at -20°C]

4.1 Buffer

10 mM PO₄
.5 mM DTT

[pH = 7.4]

X10 Sucrose Buffer

1.5 M NaCl
40 mM PO₄
2 mM MgCl₂
5 mM DTT
100 mM Tris
1.0 mM ATP
50 mM KCl

[store at -20°C]

B. Polymerize Actin

1. Take Actin frozen at -20°C in glycerol and dialyze X3 in Buffer A.
2. Make Actin 50 mM KCl, 2 mM MgCl₂.
3. Let sit 2 hrs at room temperature.

C. Mixes a la Wolfe

| | |
|--------------------|--|
| <u>S-A-4.1 Mix</u> | |
| F Actin | 240 \ of OD ₂₈₀ = 2.0 in Buffer A |
| 4.1 | 600 \ of OD ₂₈₀ = 0.2 in 4.1 Buffer |
| BSA | 20 \ of 35% Sterile |
| HS Buffer | <u>160 \</u> |
| | 1020 \ |

| | |
|------------------|--|
| <u>S.-A. Mix</u> | |
| F Actin | 240 \ of OD ₂₈₀ = 2.0 in Buffer A |
| 4.1 Buffer | |
| BSA | 20 \ of 35% Sterile |
| HS Buffer | <u>160 \</u> |
| | 1020 \ |

| | |
|----------------|---------------------|
| <u>S Mix</u> | |
| F Actin Buffer | |
| 4.1 Buffer | |
| BSA | 20 \ of 35% Sterile |
| HS Buffer | <u>160 \</u> |
| | 1020 \ |

D. The Assay

- | <u>SA 4.1</u> | <u>SA</u> | <u>S</u> |
|--|---|---|
| 1. 50 \ of SA 4.1 mix | 50 \ of SA mix | 50 \ of S mix |
| 2. 2-40 ug ¹²⁵ I spectrin dimer up to 30 \ in A-15 Buffer | 2-40 ug ¹²⁵ I spectrin dimer up to 30 \ in A-15 Buffer | 2-40 ug ¹²⁵ I spectrin dimer up to 30 \ in A-15 Buffer |
| 3. A-15 Buffer | A-15 Buffer | A-15 Buffer |
| ----- total vol = 80 \ | ----- total vol = 80 \ | ----- total vol = 80 \ |
- Incubate at 23°C 60-90 min.
 - Layer 60 \ of each sample above 5% sucrose in sucrose buffer leaving an air space in polyethylene tubes.
 - Centrifuge 2 hrs at 20 K rpm in SS34.
 - Freeze tubes and clip 7 mm from bottom. Count supernatant and pellet.

PROTEIN 4.1 PREPARATION BY TWEEN 20

All done at 4°C in SS34 rotor in RC-2B or RC-5B unless otherwise indicated.

A. Make ghosts.

B. Band 6 extraction (Glyceraldehyde-3-phosphate dehydrogenase)

1. Stir membranes in:
0.155 M NaCl = 9 g/l
0.5 mM EGTA = 2.5 ml/l of 200 mM stock
5 mM Na phosphate, pH 8
for 30 min on ice, i.e. add salt and EGTA to ghosts.
2. Centrifuge at 20 K rpm for 10 min.
3. Wash with PBS and recentrifuge.
4. Wash with glycine buffer (part C) and recentrifuge.

C. Spectrin and actin (Band 5) extraction

1. Suspend membranes in 6 vols of: (for unit bring vol to 1 l)
5 mM glycine = 1.13 g/3 l
0.5 mM EGTA = 7.5 ml of 200 mM stock/3 l
5 mM beta-mercaptoethanol = 1.07 ml/3 l
3 mM azide = .585 g/3 l
pH to 9.5
2. Stir on ice 6-12 hrs and spin at 20 K rpm for 20 min.
3. Repeat extraction 2 more times.
4. Suspend membranes in 6 vols of: (for unit bring to 1 l)
0.6 mM Na Azide = 0.78 g/2 l
0.5 mM EGTA = 5 mls of 200 mM stock/2 l
pH to 8
5. Stir on ice 6-12 hrs and spin at 20 K rpm for 20 min.
6. Repeat Azide extraction once more and recentrifuge.

D. Tween 20 4.1 Extraction

1. Dilute membrane with an equal volume of:
0.2 M glycine 7.5 g
2% Tween 20 10 cc
2 mM Na tetrathionate .306 g
1 mM EGTA 2.5 ml of 200 mM stock
pH 9.8 500 ml

2. Stir on ice 10-15 hrs.
3. Spin at 39 K rpm for 1 hr in 60 Ti rotor in Sorval or Beckman ultracentrifuge.

E. Purification at 4.1

1. Layer sample on DE52 column
(.15 ml DE52/mg protein) equilibrated in:

| | |
|-----------------------|------------------------|
| 0.05 M glycine | 3.75 g |
| 0.5 mM EGTA | 2.5 cc of 200 mM stock |
| 0.5 mM dithiothreitol | <u>.08 g</u> |
| pH 9.8 | <u>1 liter</u> |
2. Wash with equilibration buffer until OD₂₈₀ falls to 0.05 or lower using white-white tubing (36 cc/hr).
3. Apply gradient
4. Collect using black-black tubing (19cc/hr).
5. Collect 10 min fractions.
6. Run on SDS-PAGE gels to check purity.

METHOD FOR RBC LIPID EXTRACTION

DO EVERYTHING IN DUPLICATE

1. Wash anticoagulated RBC three times in saline, remove WBC with cellulose acetate columns. Final WBC should be less than $400/\text{mm}^3$.
2. Extract approximately 0.2 cc of packed RBC and determine RBC count on Coulter.
3. Use disposable 16 x 150 mm glass tubes to do the extract.
4. Add 0.5 cc distilled water to hemolyse samples. Vortex. After lysis is completed, place samples in ice bath and let cool for 5-10 minutes.
5. Add 5.5 cc of cold isopropanol to lysate and vortex. The isopropanol must be cold or the lipids will not be completely extracted from the RBC. After approximately 15 minutes in ice, let samples remain at room temperature with frequent vortexing for approximately 1 hour. The RBC membranes should look fluffy and will settle by this time.
6. Place the samples back in the cold ice bucket and add 3.5 cc COLD chloroform. Again vortex, remove from the cold in 15 minutes, and keep at room temperature for 1 hour. Frequent vortexing is necessary.
7. After 1 hour, centrifuge at $\sim 1,5000 \times g$, filter supernatant with glass wool pipets into 16 x 150 mm tube, and wash pellet.
8. Remove inorganic phosphorus by adding 2 cc .5M KCl/10 cc extract. Vortex and spin down. Remove aqueous layer (top). Repeat 2 more times. If doing just cholesterol assay skip this step.
9. Dry down extract with nitrogen in 40-45°C bath.
10. Bring up to known volume (9.25 cc) with chloroform/methanol 2/1 and aliquot for assay.

NOTE: All glassware must be phosphorus free for phosphorus assay. No rubber stoppers should be used for cholesterol assay. They are not recommended for phosphorus either.

May stop at 2 points:

- 1) after addition of isopropanol
- 2) after addition of chloroform and store at -20°C overnight.

METHOD FOR PHOSPHORUS DETERMINATION

1. Reagents: Perchloric Acid 69-72% as purchased from J.T. Baker.
Ammonium Molybdate 2.5%. Dissolve 2.5 g per 100 ml water.*
Ascorbic Acid 10% (Purchased from Fisher). Dissolve 10.0 g per 100 ml water.

*Keep at 4°C in dark bottle, make 500 cc, storage okay for 1 month.

2. Tubes used: 16 x 150 mm disposable borosilicate glass culture tubes.

3. Specimen:

Plasma: Make lipid extract from 0.2 ml plasma or serum.
Use 1.0 ml extract for phosphorous determination.

RBC: After washing in isotonic saline 3X and removing WBC's, use 1 cc at 20% Hct, spin and discard supernatant or use 0.3 cc red cells from 80% Hct to make lipid extract.
Use 1.0 of the extract for phosphorous determination.

4. Procedure:

1. Evaporate all aliquoted extracts to dryness with N₂ in 40-45°C H₂O bath.

2. Add 0.5 ml perchloric acid to each.

3. Heat in 160°C heating block for 30 min; if samples are not clear, heat longer.

4. Let cool ~5 min.

5. Add 3.3 ml H₂O to each.

6. Add 0.5 ml ammonium molybdate and vortex.

7. Add 0.5 ml ascorbic acid and vortex.

8. Boil in 100°C water bath for 10 min; put marbles on top of tubes.

9. After cooling ~10 min read at 797 nm.

5. Standard Curve:

Make .8650 g Na₂HPO₄ · 7H₂O stock solution = 100 ug P/cc.
Make working stocks of 1 ug/ml, 2 ug/ml, 3 ug/ml from above stock.
For daily standard curve, use 1 ug, 2 ug, 3 ug by drying 1 ml from each of the above working stocks in oven.
These may be dried in a batch and stored at room temp indefinitely.

Alt: Pre-ashed phosphorus STD may be purchased.

NOTE: All glassware must be new or acid washed.

TOTAL CHOLESTEROL

Method of Zlatkis

REAGENTS

1. Standard Cholesterol solution (1 mg/ml).
 - a. Dissolve 100 mg pure, dry, ash-free cholesterol in 100 ml glacial acetic acid.
 - b. Alternative: Purchase Sigma's Chol. Standard Solution in Glacial Acetic Acid (1 mg/ml).
 - c. Daily set up fresh standard curve with standards of 50 ug, 75 ug, 100 ug.
2. $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ Solution: 10 g/100 ml HAc (#2).
Make fresh daily.
3. Color Reagent (#3): Slowly dilute 1 ml of #2 to 100 ml with conc. H_2SO_4 .

PROCEDURE

1. Use either 0.2 ml plasma or 0.2 cc packed RBC. Perform isop/chlor lipid extract as indicated.
Use 3.0 ml of the extract for cholesterol determination. Use 16 x 150 mm borosilicate glass tubes. Evaporate extract to dryness with N_2 in H_2O bath at 40-45°C.
2. Add 3 ml HAc to sample tubes. Vortex 30 sec.
3. Add 2.0 ml Color Reagent (#3), slowly down the side of the tube.
This will form two layers.
Vortex 1 min to mix very well.
4. Let tubes come to RT (30 min).
5. Read at 560 nm in same order as added Color Reagent.

UNOPETTE TEST 5830



BECTON-DICKINSON
A DIVISION OF B-D

RBC OSMOTIC FRAGILITY DETERMINATION FOR MANUAL METHODS

INFORMATION AND PROCEDURE

PRODUCT INFORMATION

USE

UNOPETTE Test 5830 is a stable *in vitro* diagnostic reagent system for the determination of erythrocyte osmotic fragility, using 10 buffered saline concentrations equivalent to 0.85%, 0.65%, 0.60%, 0.55%, 0.50%, 0.45%, 0.40%, 0.35%, 0.30%, and 0.00% sodium chloride. These 10 saline concentrations are contained in UNOPETTE Reservoirs correspondingly labeled 05, 65, 60, 55, 50, 45, 40, 35, 30, and 00.

SUMMARY

This method is based on an adaptation of the original erythrocyte osmotic fragility procedure using various concentrations of sodium chloride, as described by Dacie,¹ and modified by the addition of a phosphate buffering system to maintain pH stability.² Thimerosal is added to inhibit bacterial growth.

PRINCIPLE

Whole blood is added to the buffered saline diluents which have concentrations equivalent to 0.00% to 0.85% sodium chloride.

Erythrocytes suspended in the buffered diluent equilibrate to isotonic (0.85%) saline will retain their shape and will not hemolyze. Erythrocytes suspended in the hypotonic saline diluents will take up water, swell, become spherical and more fragile, and eventually lyse, releasing hemoglobin into the solution.

The percent hemolysis that occurs within a 20 minute incubation period in each concentration is determined by absorbance measurements utilizing appropriate formula and plotted on a graph against the osmotic strengths of the saline diluents. In normal subjects, an almost symmetrical sigmoid shaped curve is obtained. Characteristically abnormal curves will be generated by different erythrocyte dyscrasias. (See PROCEDURE, Step 9.)

Results obtained with UNOPETTE Test 5830 compare favorably with those obtained using Dacie's method with sodium chloride alone in the diluent.^{1,2}

REAGENTS

1. UNOPETTE Reservoirs containing 3.08 ml of diluent mixture

Reservoir Labeled 00

Dibasic anhydrous sodium phosphate 0.140 gm
Monobasic sodium phosphate 0.020 gm
Thimerosal 0.100 gm
Distilled water qs to 1 liter

Reservoir Labeled 30

Dibasic anhydrous sodium phosphate 0.407 gm
Monobasic sodium phosphate 0.057 gm
Thimerosal 0.100 gm
Sodium chloride 2.700 gm
Distilled water qs to 1 liter

Reservoir Labeled 35

Dibasic anhydrous sodium phosphate 0.474 gm
Monobasic sodium phosphate 0.067 gm
Thimerosal 0.100 gm
Sodium chloride 3.150 gm
Distilled water qs to 1 liter

Reservoir Labeled 40

Dibasic anhydrous sodium phosphate 0.542 gm
Monobasic sodium phosphate 0.076 gm
Thimerosal 0.100 gm
Sodium chloride 3.600 gm
Distilled water qs to 1 liter

Reservoir Labeled 45

Dibasic anhydrous sodium phosphate 0.610 gm
Monobasic sodium phosphate 0.086 gm
Thimerosal 0.100 gm
Sodium chloride 4.050 gm
Distilled water qs to 1 liter

Reservoir Labeled 50

Dibasic anhydrous sodium phosphate 0.678 gm
Monobasic sodium phosphate 0.095 gm
Thimerosal 0.100 gm
Sodium chloride 4.500 gm
Distilled water qs to 1 liter

Reservoir Labeled 55

Dibasic anhydrous sodium phosphate 0.745 gm
Monobasic sodium phosphate 0.105 gm
Thimerosal 0.100 gm
Sodium chloride 4.950 gm
Distilled water qs to 1 liter

Reservoir Labeled 60

Dibasic anhydrous sodium phosphate 0.813 gm
Monobasic sodium phosphate 0.114 gm
Thimerosal 0.100 gm
Sodium chloride 5.400 gm
Distilled water qs to 1 liter

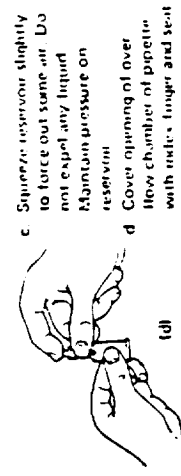
Reservoir Labeled 65

Dibasic anhydrous sodium phosphate 0.881 gm
Monobasic sodium phosphate 0.124 gm
Thimerosal 0.100 gm
Sodium chloride 5.850 gm
Distilled water qs to 1 liter

Reservoir Labeled 85

Dibasic anhydrous sodium phosphate 1.152 gm
Monobasic sodium phosphate 0.162 gm
Thimerosal 0.100 gm
Sodium chloride 7.650 gm
Distilled water qs to 1 liter

2. 10 UNOPETTE Capillary Pipettes 20 µl capacity



c. Squeeze reservoir slightly to force out some air. Do not expel any liquid. Maintain pressure on reservoir.

d. Cover opening of overflow chamber of pipette with index finger and seal pipette securely in reservoir neck.

e. Release pressure on reservoir. Then remove finger from pipette opening. Negative pressure will draw blood into diluent.

f. Squeeze reservoir gently two or three times to mix capillary bore, forcing diluent up into, but not out of, overflow chamber, releasing pressure each time to return mixture to reservoir.

CAUTION See WARNING Section

g. Place index finger over upper opening and gently invert several times to thoroughly mix blood with diluent.

Repeat PROCEDURE 2 (a-g) for all ten labeled reservoirs using a new pipette for each dilution.

3. INCUBATE Incubate diluted samples at room temperature for twenty (20) minutes.

4. TRANSFER CONTENTS Transfer thoroughly mixed contents of each reservoir to appropriately labeled test tube as follows:

- Convert to dropper assembly by withdrawing pipette from reservoir and reseat securely in reverse position.

b. Place capillary tip into appropriately labeled test tube and squeeze reservoir to expel entire contents.

5. CENTRIFUGE

Centrifuge all samples at 2000 rpm for five (5) minutes.

6. TRANSFER CONTENTS

Keeping samples in proper sequence, pour off supernate from test tubes into correspondingly labeled cuvettes which accommodate 4.0 ml of fluid. Be careful not to decant centrifuged cell button.

7. SET WAVELENGTH

Set the wavelength on a spectrophotometer to 660 nm or use a filter photometer with appropriate filter.

8. READ ABSORBANCE



a. Wipe outside of cuvettes before placing in instrument.

b. Check zero stability of instrument using cuvette filled with distilled water to act as Blank.

c. Measure absorbance of all ten (10) Unknown concentrations against Water Blank set at zero absorbance, and record.

9. CALCULATE

Determine percent hemolysis in each specimen by substituting absorbance value (specimen density IOD I) for the desired concentration in the following formula:

$$\% \text{ Hemolysis} = \frac{\text{IOD} - \text{IOD}_{\text{Blank}}}{\text{IOD}_{\text{Blank}} - \text{IOD}_{\text{Water Blank}}} \times 100$$

IOD = Absorbance of solution of any known osmotic strength of sodium chloride. 0.65 ± 0.005 to 0.55 ± 0.005 0.45 0.30 0.25 0.20

EXAMPLE:

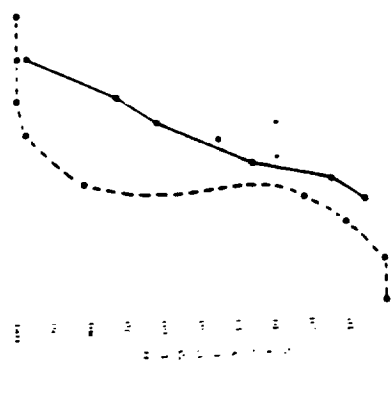
Absorbance (IOD) values obtained and percent hemolysis calculated as follows:

| Cuvette Label | Osmotic Strength Sodium Chloride | IOD |
|---------------|----------------------------------|-------------|
| (W) | 0.00 | 0.305 |
| 45 | 0.45 | 0.218 |
| 85 | 0.85 | 0.005 |
| Hemolysis | 0.218 0.005 X 100 | % Hemolysis |
| | 0.305 0.005 | |

10. CONSTRUCT ERYTHROCYTE FRAGILITY GRAPH

Plot percent hemolysis values on the abscissa against osmotic strength of saline diluents on the ordinate. First point on the ordinate must be 0.85.

Typical Erythrocyte Fragility Graph with Normal Limits Defined and Two (2) Dyscrasias Illustrated.



Osmotic Strength of Saline Diluents
0.85 0.65 0.60 0.55 0.50 0.45 0.40 0.35 0.30 0.25 0.20

LIMITATIONS OF PROCEDURE

If specimen of low hemoglobin content is to be tested, wash erythrocytes in the specimen and in the control sample in isotonic saline. Centrifuge, decant supernate and resuspend both samples so that the ratio of cells to saline is the same for both. Then proceed with analysis.

EXPECTED NORMAL VALUES

| Reservoirs Labeled | Osmotic Strength of Sodium Chloride Solution | Approximate Hematocrit |
|--------------------|--|------------------------|
| 00 | 0.00% | 100 |
| 30 | 0.30% | 97.100 |
| 35 | 0.35% | 90.99 |
| 40 | 0.40% | 50.90 |
| 45 | 0.45% | 5.45 |
| 50 | 0.50% | 0% |
| 55 | 0.55% | 0 |
| 60 | 0.60% | 0 |
| 65 | 0.65% | 0 |
| 85 | 0.85% | 0 |

EXPECTED PERFORMANCE

Precision of Values of Erythrocyte Fragility Test on Normal Blood in 101 using UNOPETTE 101-5030

| Reservoir Labeled | Osmotic Strength of Sodium Chloride Solution | Approximate Hematocrit |
|-------------------|--|------------------------|
| 00 | 0.00% | 100 |
| 30 | 0.30% | 97.100 |
| 35 | 0.35% | 90.99 |
| 40 | 0.40% | 50.90 |
| 45 | 0.45% | 5.45 |
| 50 | 0.50% | 0 |
| 55 | 0.55% | 0 |
| 60 | 0.60% | 0 |
| 65 | 0.65% | 0 |
| 85 | 0.85% | 0 |

TECHNICAL NOTES

- It is advisable to run normal blood as a control when testing an unknown sample for erythrocyte fragility.

- A fresh sample is generally desirable for this procedure because cell shape and osmotic balance change with time.
- Incubation period should be conducted at room temperature. If fluctuations in temperature exist within the work area, reservoirs should be incubated in a room temperature water bath for consistent results.
- To discuss certain subtle abnormalities in erythrocyte osmotic fragility, delaminated or hemolyzed blood is incubated *in vitro* under sterile conditions at 37°C for 24 hours. Consult Reference 7 for technique.
- If visible hemolysis is present in 0.5% or less osmotic solution, the determination should be repeated using a fresh specimen.

REFERENCES

1. B. D. Becton-Dickinson, "Erythrocyte Fragility Test," *Journal of Clinical Investigation*, 30: 1000-1005 (1951).
2. B. D. Becton-Dickinson, "Erythrocyte Fragility Test," *Journal of Clinical Investigation*, 30: 1000-1005 (1951).
3. B. D. Becton-Dickinson, "Erythrocyte Fragility Test," *Journal of Clinical Investigation*, 30: 1000-1005 (1951).
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5. B. D. Becton-Dickinson, "Erythrocyte Fragility Test," *Journal of Clinical Investigation*, 30: 1000-1005 (1951).
6. B. D. Becton-Dickinson, "Erythrocyte Fragility Test," *Journal of Clinical Investigation*, 30: 1000-1005 (1951).
7. B. D. Becton-Dickinson, "Erythrocyte Fragility Test," *Journal of Clinical Investigation*, 30: 1000-1005 (1951).

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BIOLOGICAL RESEARCH DEPARTMENT
BECTON-DICKINSON COMPANY
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14.2 Platelet Studies

Sample Aliquoting and Designated Responsibility for the Platelet Study.

Persons Involved:

D. Ausprunk (D.A.); W. Curby (W.C.); M. Jacobson (M.J.); I. Szymanski (I.S.); A. England (A.E.); Lisa Rice (L.R.); B. Kim (B.K.); D. Kenney (D.K.); F. Chao (F.C.).

| Tests | Responsible Person | Blood Volume(ml) | Performance Site | Reference Section in Original Proposal |
|------------------------------------|--------------------|------------------|---------------------|--|
| pH viscosity | M.J. | 1 | on site | 4.2.2.1 |
| platelet count | W.C. | 1 | on site | 4.2.2.1 |
| volume | | | | 4.2.2.2 |
| size | | | | |
| shape | | | | |
| morphology & shape change | D.A. | 7 | on site | 4.2.2.2 4.2.2.3 |
| platelet aggregation & ATP release | B.K. & A.E. | 4 | on site | 4.2.2.4 4.2.2.7 |
| Thromboxane generation | L.R. | 2 | on site & in Boston | 4.2.2.5 |
| uptake & release of 5 HT | F.C. | 2 | on site & in Boston | 4.2.2.6 |
| β -thromboglobulin release | A.E. | 2 | on site & in Boston | 4.2.2.7 |
| PF-3 measurement | F.C. & L.R. | 3 | on site | 4.2.2.8 |
| Hyp otonic Stress | B.K. | 3 | on site | 4.2.2.9 |
| membrane glycoproteins | D.K. & F.C. | 15 | on site & in Boston | 4.2.2.10 |
| cytoskeleton | D.K. & F.C. | 15 | on site & in Boston | 4.2.2.11 |
| Immunology | I.S. | 3 | on site | |

Assays of Platelet Aggregations and Release Reaction

A. Working Space

50" x 25" bench space

4 plugs of the power line with 115 V AC, 60 Hz

B. Equipment and Accessories:

1. Lumi-Aggregometer (Model 1020, Payton Scientific, Inc., Buffalo, NY)
2. Two Dual Channel Strip Chart Recorders (Model 500, Payton Sci., Inc., Buffalo, NY).
3. Chart Paper (#0100-025, Payton Sci., Inc., Buffalo, NY); one roll
4. Cuvettes, disposable (CU312, Payton Sci., Inc., Buffalo, NY); two boxes
5. Stirring Bar, (SB312, Payton Sci., Buffalo, NY); 100 bars
6. Pipettes, 1 ml, 200 ul, 10 ul capacities; two each
7. Pipette tips, blue and yellow

C. Reagents:

1. ADP stock solution, 10^{-3} M in 0.87% NaCl, 10 ml in total
2. Collagen stock solution, 20 mg %, 5 ml in total
3. ATP standard solution, 10^{-3} M, 3 ml in total
4. Luciferase-Luciferin Reagent (Part #395, Chrono-Log Co.); four vials

D. Assay Procedures:

1. Preparation of samples:
Platelet concentrate of 3 ml aliquot sampled from each storage unit will be adjusted to platelet count 300,000 per ul by diluting with pooled citrate plasma. For this, 60 ml of pooled plasma will be obtained of which 10 ml will be used for control samples (before storage; to) and 50 ml for stored samples. The plasma for stored samples will be kept at 4°C until used.
2. Simultaneous assays for aggregation and release reaction:
Platelet samples of 0.4 ml plus the luciferase-luciferin reagent of 0.05 ml will be prewarmed to 37°C in the incubator of lumiaggregometer. The recording chart will be adjusted to a full scale with the sample and plasma as 0% and 100% light transmittance, respectively. The assay will be started by adding aggregating agent of 0.05 ml into stirring sample. Aggregating pattern and the intensity of luciferin light produced by the released ATP will be recorded in a dual channel recorder. At the end of reaction (usually 4-5 mins), 2 ul of ATP standard solution containing 2 nmol will be injected and light intensity will be recorded and used as an internal standard for calculation. Two different concentrations of both aggregating agents, ADP (10 uM and 100 uM) and collagen (6.25 ug and 12.5 ug per assay) will be tested for each sample. Maximal degree of aggregation will be recorded and the value of release reaction will be expressed in umol ATP released from the aggregating platelets, e.g. per 10^{11} cells.

UPTAKE AND RELEASE OF SEROTONIN

Equipment:

| | |
|------------------|-----------------------|
| Microfuge | pH meter |
| Vortex | Scintillation Counter |
| Water Bath 37° C | Cell counters |
| Pipettes | Heater |

Materials:

| | |
|------------------------------|---|
| Distilled H ₂ O | pooled plasma; platelet concentrates |
| NaCl | TES |
| EDTA | HCl |
| Horm collagen | ¹⁴ C - 5HT |
| Protozol | NaOH |
| plastic tubes for incubation | Scintillation fluid |
| plastic vials for counting | microfuge tube, polycarbonate outer tubes |

Plasma preparation:

Dilute platelet concentrates with pooled plasma to 300,000 cells/ul.

Solutions:

1.2% paraformaldehyde solution (PFA)

1. Dissolve 6 gm of PFA in 100 ml of distilled H₂O; heat to 60-70°C; add 5N NaOH until solution becomes clear.
2. Dilute (1 part) 6% formalin solution with 5 parts of TES-saline-EDTA (0.01 M TES, 0.14 M NaCl, 5 mM EDTA; pH 7.4).
3. Adjust pH to 7.4 if necessary by adding HCl.

Collagen solution (Horm Collagen)

Dilute collagen stock (100 ul) with 400 ul buffer (conc. 200 ug/ml).

Procedure:

1. Mix PRP (2 ml, 300,000 cells/ul) with ¹⁴C-5HT (final conc. 5 uM) and incubate at 37°C.
2. At 1, 3, 5, 10 and 30 min. after incubation, remove 150 ul of the reaction mixture and put in microfuge tubes (hard tube); centrifuge at 12000 rpm for 2 min. in a microfuge.
3. Remove 50 ul (x2) of supernatant plasma and add 0.2 ml of protozol. Incubate overnight. Add scintillation fluid and count.

4. Remove 50 ul of PRP in duplicates and mix with 0.2 ml of protozol to determine total radioactivity.
5. To the remainder of PRP, add 10 ul of diluted collagen (final conc. 2 ug/ml), mixed by vortex.
6. At 1, 3, 5, 10 and 15 min after addition of collagen, 100 ul of the reactive mixture will be removed and added to 400 ul of 1.2% paraformaldehyde solution.
7. Centrifuge at 12000 rmp for 2 min. in a microfuge, remove 50 ul of supernatant, mix with 0.2 ml of protozol overnight and 5 ml of Liquiscint solution (National Diagnostics; Liquiscint will be acidified by mixing 10 ul of glacial acetic acid with 1.5 ml of Liquiscint prior to use). The samples will be counted for radioactivity in a liquid scintillation counter using an established counting program.

SAMPLE PREPARATIONS FOR β -TG AND TxB MEASUREMENT BY RIA

1. Gently add 2.5 ml of platelet concentrates to the blood collection tubes (which contain anticoagulant and antiplatelet agents) provided with the radioimmunoassay kit. The tubes are pre-marked at 2.5 ml.
2. Stopper the tubes and mix the content by gentle inversion two or three times.
3. Cool the sample by placing the blood collecting tubes in cooling bath for 15 min.
4. Centrifuge the sample at 1500-2000 g at 2-4°C for 30 min.
5. Remove the top 0.5 ml of plasma using a pipette with a disposable plastic tip.
6. Transfer the sample to a separate labeled specimen tube.
7. Store the sample at -20°C and transport the sample back to Boston for assay.

Storage conditions: 24 hrs at room temperature
 7 days at 2-4°C
 4 weeks at -20°C

8. See attached package inserts.

INITIAL BLOOD STORAGE EXPERIMENT - PLATELETS

PF3-1

PF-3 Measurements

Equipment:

| | |
|------------------------------|--------------|
| Microfuge | Wire loop |
| Congulatron assay water bath | Ice buckets |
| Stop watches | Cell counter |

Materials:

| | |
|---|-------------------------------|
| Platelet concentrates | Distilled water |
| glass clotting tubes | CaCl ₂ , TES, NaCl |
| Russell's viper venom | Pipette |
| Microfuge tube (hard tubes) | Ice |
| PPP prepared from platelet concentrates | |

Solutions:

1. Russell's viper venom, working stock 10 ug/ml in TES-saline.
2. CaCl₂. 0.05 M CaCl₂ in H₂O.

Platelet and plasma preparations:

1. Centrifuge 2 ml of platelet concentrate at 12,000 rpm at room temperature for 2 min to obtain PPP.
2. Save 0.5 ml of PPP for PF-3 measurement.
3. Dilute platelet concentrate with PPP to obtain PRP at cell count of 300,000/ul.
4. During preparation of PPP and PRP, the tubes will be capped.

Procedures:

1. Prewarm CaCl₂ solution at 37° C.
Store PRP and PPP at room temperature.
Keep RVV on ice.
2. Add 0.1 ml of PRP or PPP into a glass clotting tube.
Add 0.1 ml of CaCl₂, incubate at 37° C for 1 min.
3. Add 0.1 ml of RVV. Start the stop watch.
4. Mix the reaction mixture constantly with a wire loop until a film clot forms over the loop; stop the watch; record the time.

Assay of Platelet Response to Hypotonic Stress (PRHS)

A. Working Space:

65" x 25" bench space
2 plugs of the power with 115 V AC, 60 Hz

B. Equipment and Accessories

1. Spectrophotometer (Beckman DU, Model #2400)
2. Strip Chart Recorder (Omni-Scribe, Model #B-5000)
3. Chart Paper (Cat. #13-939-35, Fisher Sci. Co.); one roll
4. Cuvettes, semi-micro uv cell, 1 ml capacity; one set of four
5. Pipettes; two 1 ml and one 0.2 ml capacity
6. Pipette tips; blue and yellow
7. Other facilities for platelet countings (Coulter count, sample diluting vials and Isotonic diluent, etc.)

C. Reagents: NONE

D. Assay Procedures:

1. Preparation of samples:
Platelet concentrate of 2 ml aliquot sampled from each storage unit will be adjusted to platelet count 400,000 per μ l by diluting with pooled donor plasma. The pooled plasma of 50 ml will be needed, 5 ml for the to sample and 45 ml for the stored samples.
2. Assay:
Platelet sample of 0.5 ml will be admixed rapidly with an equal volume of distilled water in the cuvette and the change of optical density of the platelets will be recorded in a spectrophotometer at the light wave length of 420 nm. A sudden drop in optical density occurs by exposing the platelets to a hypotonic condition which is followed by a gradual return to the original density. The latter phase, an increase in optical density units during the first 2 minutes, will be the value of the PRHS test.

Introduction of ^{125}I into Tyrosines on Platelet Membrane Proteins and Glycoproteins by Lactoperoxidase Catalyzed Radioiodination

Literature: Modified from Phillips, D.R., Biochem. 11:4582-4588, 1972;
Phillips, D.R. & Agin, P.P., J. Biol. Chem. 252:2121-26, 1977;
Holihan, J.R. & White, G.C., Blood 57:174-181, 1981.

REAGENTS:

1. H_2O_2 -Fisher Cat. #H-325; 30% stock (8.8 M) diluted 1:1000 into cold (4°C) PBS immediately before use. Stock is kept refrigerated and discarded approximately 1 month after opening.
2. Lactoperoxidase - Cal. Biochem Cat. #427488; dissolve in PBS pH 7.4 at 200 ug or .2 U/ml store frozen at -20°C in 600 ul aliquots. Molecular weight 80,000.
3. Na^{125}I - Amersham Cat. #IMS-30; purchased in 1 mCi quantities - takes 1-2 days for delivery after order; store at room temp. in radioactive area.
4. 10 mM KI - prepared as a stock, kept frozen in aliquots.
5. Solution of TES/Tyrode's with 5 mM EDTA + glucose and 200 uM KI.
6. Solution of TES/Tyrode's with 5 mM EDTA + glucose and 100 uM KI.
7. Solution of TES/Tyrode's with 5 mM EDTA, no glucose, no KI.
8. Solution of 1% Nonidet P40 in TES/Tyrode's with 5 mM EDTA (no glucose), 5 mM Iodoacetamide, .2 mM PMSF.

EQUIPMENT:

| | |
|---|--------------|
| Hood equipped for radioiodination | Vortex |
| Intermediate Speed Centrifuge | pH Meter |
| Facilities for disposal of ^{125}I waste | Cell Counter |

PROCEDURE FOR 5 ml LABELING-REACTION MIXTURE:

1. Count washed platelets and adjust concentration to $0.5 \times 10^9/\text{ml}$ (range $3-7 \times 10^8/\text{ml}$) in 5 ml.
2. Bring solution to 10 uM KI (5 ul of 10 mM/5 ml).
3. 2.5 ul of NaI^{125} (or 250 uCi).
4. Add 1.25 nMoles LPO (500 ul of frozen stock).
5. H_2O_2 diluted in ice-cold PBS just before use.

6. Add H₂O₂ at 30 sec. intervals in 5,6 ul aliquots. Platelet suspension is stirred constantly on a magnetic stirrer with Fisher microbar (Cat. No. 14511-69) magnet.
7. Dilute to 35 ml with TES/Tyrodes, 5 mM EDTA, + 200 uM KI.
8. Pellet in IEC centrifuge at 2,000 rpm for 10 min at room temp.
9. Resuspend pellet in 15 ml TES/Tyrodes with 5 mM EDTA + 100 uM KI.
10. Pellet in IEC at 2,000 rpm for 10 min at room temp.
11. Resuspend in TES/Tyrodes/EDTA (no glucose) at a final concentration of 1.0×10^9 /ml (cells must be counted).
12. Extract platelets for 5 min at room temp. with 0.5% NP40 by adding an equal volume of TES/Tyrodes + 5 mM EGTA containing 5 mM iodoacetamide and 1% NP40.
13. Freeze at -20°C for electrophoretic analysis.

Analysis of ¹²⁵I-labeled Membrane Proteins (Boston)

- A. One dimensional SDS Polyacrylamide Gel Electrophoresis (SDS-PAGE) will allow detection of gross changes (i.e., cleavage, loss from membrane) in major platelet surface glycoproteins, particularly gpIIb and gpIIIa. Samples will be analyzed under both reducing and non-reducing conditions to detect changes in S-S bonding and SH-groups (and effect of microgravity) of membrane proteins that occur during storage. SDS-PAGE will be carried out using the discontinuous Laemmli System (Laemmli, Nature 227:680-85, 1970).
 1. Solubilize extracts (prepared in #12 above containing 1 mg platelet protein/ml) with 1/10 volume 10% SDS, boil for 2 min at 100°C, mix with Laemmli Sample Buffer containing 1.8% SDS, 125 M Tris-HCl pH 6.8, 20% glycerol, bromophenol blue, and for analysis of proteins under reducing conditions, 5% beta-mercaptoethanol.
 2. Apply aliquots of solubilized samples containing 50-120 ug of protein to precast SDS-slab gels consisting of a 4% stacking gel and 7.5% resolving gel.
 3. Electrophorese at 24 mAmps/slab until bromophenol blue tracking dye reaches the leading edge of the gel. Run off for 45 min additional minutes at 24 mAmps to improve resolution of components in the 100-200,000 molecular weight range.
 - *4. Stain gels in 0.025% Coomassie Brilliant Blue for 2 hrs.
 5. Destain in 2-3 changes of 30% methanol/10% acetic acid overnight.
 6. Photograph wet gel using an orange filter to record profile of total platelet polypeptides.

7. Dry down gel on vacuum slab gel dryer.
8. Detect radiolabeled proteins by autoradiography using X-OMAT (Kodak) film and intensifying screens. 3-5 days exposure at -80°C will give bands of sufficient intensity.
9. Quantitation of radiolabeled proteins will be performed by densitometric scanning of autoradiograms using a Hoeffer gel scanner.

*Duplicate gels will be stained by the Alcian Blue method which selectively stains glycoproteins (Wardi and Mechow, Anal. Biochem 49:607-09, 1972).

1. Fix gels in 12.5% Trichloroacetic acid for 30 min.
2. Rinse gels 5X with distilled water then oxidize glycoproteins with 1% periodic acid.
3. Rinse 2X, with changes 30 min each distilled water.
4. Soak gels in 0.5% potassium metabisulfite for 30 min.
5. Rinse gels in distilled water.
6. Stain overnight in 1% Alcian Blue in 3% acetic acid.
7. Destain with 7% acetic acid.
8. Photograph wet gels.
9. Dry down and subject to autoradiography as described above.

B. Two-Dimensional Electrophoretic Analysis of ^{125}I -labeled surface proteins involving separation of proteins first on the basis of charge by Isoelectric Focusing then on the basis of molecular weight by SDS-PAGE. The method used is that of O'Farrell (J. Biol. Chem 250:4007, 1975) as modified by Linck et al. (Cell Motility 1:127, 1982). The analyses will permit detection of subtle changes in charge distribution of membrane proteins.

1. NP-40 extracts of ^{125}I -labeled platelets are prepared for isoelectric focusing by adding solid urea (ultrapure), Triton-X-100, dithiothreitol to final concentrations of 9.5 M, 3.2% and 100 mM respectively.
2. Isoelectric focusing gels, cast in 200 μl glass capillary pipettes, consisting of 3.5% polyacrylamide, 3.2% Triton-X-100, 4% ampholines (pH range 5-8), 0.4% ampholines (pH range 4-6), 0.6% ampholines (pH range 3.5-10), are prefocused at 200 V for 15 min, 300 V and 400 V each for 30 min. Electrode solutions are 0.02 M NaOH at the cathode and 0.01 M H_3PO_4 at the anode.
3. Samples containing 30-60 μg total platelet proteins are applied to the basic end of the isoelectric focusing gels and focused for 16 hrs at 400 V and 2 hrs at 1000 volts (8400 volt hours).

4. The isoelectric points of relevant polypeptides are determined from duplicate IEF gels electrophoresed in parallel without protein. The blank gradient gel is cut into 5 mm length and eluted with 0.5 ml deionized water; the pH of the eluted ampholines is measured with a pH meter. The pH values of eluted ampholines are plotted with respect to gel length. The isoelectric points are then correlated with the stained bands on another duplicate gel after correction for expansion of the stained/destained gel.
5. For SDS-PAGE, the IEF gel containing fractionated proteins is equilibrated for 20 min at room temperature in Laemmli sample buffer containing 5% beta-mercaptoethanol and placed across a precast slab gel consisting of a 4% stacking gel and a 7.5% resolving gel.
6. Electrophoresis of the 2nd dimension SDS-PAGE is as described above for one dimensional SDS-PAGE.
7. Gels are stained with Coomassie Blue, destained and labeled proteins detected by autoradiography as described above.

³H-Labeling of Sialic Acid Residues of Platelet Membrane Glycoproteins

It is necessary to examine possible storage-induced changes in platelet surface membrane proteins (and the effects of microgravity on these changes) by both the ¹²⁵I and ³H methods since different membrane proteins become radiolabeled by these techniques. For example, platelet membrane glycoprotein Ib, the vWF receptor, which is lost from the platelet membrane during storage, labels well with ³H-NaBH₄ technique, but does not incorporate significant ¹²⁵I or stain with Coomassie Blue.

Literature:

Steiner et al., Thrombo. Res. 29: 43, 1983.

Reagents:

| | |
|---|-----------------------------|
| Modified Tyrode's Buffer containing | |
| 5 mM EDTA buffered with Hepes at pH 6.8 and 7.6 | EDTA: .25 M stock |
| [³ H] NaBH ₄ (5 mCi in 50 ul cold 0.01 M NaOH) | HEPES: 1 M stock |
| iodoacetamide - .5 M frozen stock | NP-40: 10% stock sol. (w/w) |
| sodium metaperiodate - prepared fresh as 4 mM | NaOH: .1 N stock |
| solution in Hepes/Tyrodes EDTA at pH 6.8 | |

Equipment:

| | |
|--|-------------|
| Intermediate Speed Centrifuge | Ice Machine |
| Cell counter | pH Meter |
| Fume hood | Vortex |
| Barrel for disposal of ³ H contaminated waste | |

Procedure:

1. Wash platelets twice by differential centrifugation in a modified Tyrodes' buffer containing 5 mM EDTA and buffered at pH 6.8 with 10 mM HEPES.
2. Resuspend washed platelets in 1-2 ml of the same buffer.
3. Adjust platelet count to 0.5×10^9 /ml. Equilibrate at 4°C in the dark for 20 mins.
4. Add freshly prepared 4 mM sodium metaperiodate to the cooled platelet suspension to a final concentration of 2 mM and allow the reaction mixture to stand in the dark for 10 mins.
5. Dilute platelet suspension to 15 ml with HEPES/Tyrode's/EDTA at pH 6.8 and pellet.
6. Wash platelets twice with HEPES/Tyrode's/EDTA at pH 7.6 and resuspend in 1-2 ml of the same buffer; adjust platelet count to 0.5×10^9 /ml.

7. Add [^3H] NaBH_4 (final concentration 500 $\mu\text{Ci}/0.5 \times 10^9$ platelets) to the washed platelet suspension and incubate with periodic agitation, for 30 mins at room temperature.
8. Wash radio-labeled platelets twice with HEPES/Tyrod's/EDTA pH 6.8 and resuspend in the same buffer.
9. Extract platelets with 0.5% NP-40 in the presence of 5 mM iodoacetamide for 5 mins at room temperature.
10. Store extracts of radio-labeled platelets frozen at -20°C .

Analysis of ^3H -labeled platelet membrane proteins will be performed in Boston using:

- 1) SDS-PAGE and
- 2) Two dimensional isoelectric focusing and SDS-PAGE, essentially as described in the previous section for ^{125}I -labeled membrane proteins. In the case of the ^3H -labeled samples, radiolabeled glycoproteins will be detected by fluorography using diphenyloxazole as described by Bonner and Laskey (Eur. J. Biochem. 46:83, 1974).

Cytoskeleton Studies

These experiments will analyze both qualitative and quantitative changes in the composition and assembly state of platelet cytoskeletal components.

Approach:

That percentage of the total platelet 1) actin and 2) tubulin present in the cytoskeletal fraction of platelet lysates after extraction with Triton-X-100 will be quantitated. The cytoskeletal fraction which is detergent insoluble will be harvested by selective centrifugation. Actin filament content will be determined by quantitative densitometry of Coomassie blue stained SDS-gels of electrophoretically fractionated cytoskeletons. Microtubule content will be determined as "sedimentable tubulin" also by densitometry of Coomassie blue stained SDS-gels of electrophoretically fractionated cytoskeletons but from platelets that were pre-treated with Taxol to stabilize assembled microtubules prior to extraction with Triton-X-100.

Literature:

Fox et al., J. Cell Biol. 98: 1985, 1984.
Kenney and Linck, submitted for publication, 1985.

Equipment:

Low speed centrifuge
Ultracentrifuge and/or Air fuge (Beckman Instruments)
Cell counter
37°C waterbath
60°C waterbath
boiling waterbath

Platelet Preparation for Assays Described Below:

1. Wash platelets twice in TES/Tyrode's buffer, pH 7.4 containing 10 ng/ml prostaglandin E₁, and 5.5 mM glucose maintained at 22-35°C.
2. Resuspend platelets at a final concentration of 0.5×10^9 /ml in TES/Tyrode's pH 7.2 with 5.5 mM glucose.
3. Equilibrate for 1-1.5 hours at 37°C.

Assembled Microtubules:

Reagents:

1. MT-Cytoskeleton Extraction Buffer: TES/Tyrode's pH 6.8; 12 mM EGTA; Leupeptin 10 ug/ml; 1% Triton-X-100 at 22°C.
2. Taxol: 20 mg/ml in dimethylsulfoxide stored at -20°C.
3. 27% Sucrose: in TES/Tyrode's pH 6.8 with 6 mM EGTA.
4. Solubilization Buffer: 1% SDS in 0.125 M Tris-HCl pH 6.8; 6 mM EGTA; 10 mM DTT; 0.5% Na azide.

Procedure:

1. Treat 10 ml aliquot of washed, 37°C equilibrated platelets with Taxol (4 ug/10⁸ platelets) for 2 mins at 22 ± 2°C.
2. Extract platelets by mixing with 10 ml MT-cytoskeleton Extraction Buffer for 5 mins at 22 ± 2°C.
3. Layer 20 ml extract on top of 27% Sucrose.
4. Pellet assembled microtubules at 100,000 x g for 30 min at 25°C.
5. Discard supernatant and sucrose layer.
6. Solubilize pellet (prepared from 5.0 x 10⁹ platelets) in 400 ul Solubilization Buffer, heat at 60°C for 30 min and then boil for 2 min. Store samples frozen at -20°C for analysis in Boston.
7. To determine total platelet tubulin (microtubule protein), extract a parallel 10 ml aliquot of each platelet suspension, with 10 ml extraction buffer. Add Taxol to a final concentration of 50 ug/ml of extract (assuming that each ml contains protein from .25 x 10⁹ platelets), and incubate for 30 mins at 37°C; Pellet and solubilize microtubules as described above in #4 and #6.

Actin Filament:

Reagents:

1. A-Cytoskeleton Extraction Buffer: 2% Triton-X-100; 10 mM EGTA; .1 M Tris-HCl, pH 7.4 at 22°C.
2. Solubilization Buffer: (see Microtubules).
3. 4X Solubilization Buffer: 0.48 M Tris-HCl, pH 6.8; 9.6% SDS; 40 mM DTT.

Procedure:

1. Treat 5 ml washed, 37°C equilibrated platelets with 5 ml A-Cytoskeleton Extraction Buffer at $22 \pm 2^\circ\text{C}$.
2. Pellet insoluble cytoskeletons at $150,000 \times g$ at 0°C for 45 mins.
3. Wash sedimented material with 1:1 mixture of TES/Tyrode's pH 7.4 and A-Cytoskeleton Extraction Buffer.
4. Solubilize actin cytoskeletons from 2×10^9 platelets in 200 μl solubilization buffer, boil for 2 min, then freeze at -20°C for analysis in Boston.
5. Extract 1.5 ml aliquot of each platelet suspension directly with 500 μl 4X Solubilization Buffer for determination of the total actin in platelet extract. Boil the extract for 2 min, then freeze at -20°C for analysis in Boston.

Analysis of Cytoskeleton Polypeptides

Analysis of cytoskeleton polypeptides will be carried out on polypeptides which are electrophoretically fractionated using the SDS system of Laemmli (Nature 227:680, 1970) with 4% stacking gel and 8% resolving gels.

1. Cytoskeleton pellets (and platelet extracts used for determination of total platelet tubulin and actin) are solubilized with 1% SDS, brought to a final concentration of 0.0625 M Tris HCl, pH 6.8, and the disulfide bonds reduced with 5% beta-mercaptoethanol.
2. Electrophoresis is carried out at 24 mAmps per gel for 4-5 hrs.
3. Gels are stained with 0.025% Coomassie Brilliant Blue and destained with 30% methanol/10% acetic acid and photographed.
4. Molecular weights of cytoskeletal polypeptides are determined based on migration of the standard polypeptides: spectrin heterodimer 240,000 and 220,000; beta galactosidase, 130,000; phosphorylase a, 94,000; Bovine serum albumin, 68,000; glutamate dehydrogenase, 56,000; creatine kinase, 40,000 and carbonic anhydrase, 29,000.
5. Major polypeptides of the platelet cytoskeleton to be examined for qualitative and quantitative changes are: Actin binding protein, 260,000; Myosin, 200,000; alpha actinin, 90,000; Tubulin alpha, 58,000; Tubulin beta, 53,000 and actin, 43,000.
6. Amounts of individual polypeptides in cytoskeletons and supernatant fractions will be quantitated by densitometry of Coomassie Blue Stained gels using a Helena Quick Scan Densitometer with a 570 nm filter. Mean values will be determined from three scans of each sample.
7. Percent of the total cytoskeletal protein which is assembled in organized cytoskeletal structures will be determined by quantitating that particular polypeptide in the insoluble cytoskeletal fraction relative to the total

quantity of that platelet protein. It is anticipated that during storage 1) the content of cytoskeleton associated microtubule protein will decrease and 2) the content of cytoskeleton associated actin will increase.

Viscosity

Viscosity determinations will be made on a modified cone plate Wells-Brookfield microviscometer. The modifications facilitate measuring shear stress at low shear rates. The cone and plate contain radial etchings 60 microns deep to reduce the plasma layer effect at the containing surfaces. All determinations are made at 37°C. Viscosity will be measured at shear rates from 45-2.2 sec⁻¹. Each determination requires 0.5 cc sample and is done in duplicate. The instrument is standardized with samples of known viscosity obtained from Wells-Brookfield.

THE COLORIMETRIC DETERMINATION OF GLUCOSE

REF: Sigma Technical Bulletin No. 635

REAGENTS:

- A. O. Toluidine Reagent, Stock No. 635-6
Store in dark at room temperature.
Caution: Do not pipet by mouth. Avoid contact with skin.
Do not inhale fumes.
- B. Trichloroacetic Acid Solution, 3% (W/V), Stock No. 635-3
Store in refrigerator at 0-5°C.
- C. Glucose Standard Solution, Stock No. 635-100
Standardized at 1.0 mg/ml (100 mg/100 ml or 5.5 m mol/l) with
Benzoic Acid added as preservative
Store in refrigerator at 0-5°C.
- D. Hyland Q-Pak--Chemistry Control Serum 1
List No. 045-030
Store between 2° and 8°C before and after reconstitution.

DEPROTEINIZATION OF PLATELET CONCENTRATE:

Into a centrifuge tube, pipet:
0.4 ml sample
3.6 ml of 3% Trichloroacetic Acid, Stock No. 635-3
Mix well by shaking. Allow to stand approximately 5 minutes to precipitate proteins.
Centrifuge (5-10 minutes) until clear supernatant is obtained in the RC-3 at 3000 rpm. Store clear supernatant at 0-5°C, if determination is to be within a few hours. Otherwise freeze clear supernatant.

INSTRUMENT:

Practically any photoelectric colorimeter that transmits light in the range of 620-650 nm can be used.

Instrument: Coleman Jr.
Wavelength: 635 nm
Cuvette: 19 x 150 mm

NOTE: Instrument readings should be made in terms of Absorbance (A) for use in calculations and for preparation of calibration curves.

ASSAY PROCEDURE:

1. Label test tubes or cuvetts BLANK, STANDARD, CONTROL, TEST 1, TEST 2, etc.

| <u>TO BLANK ADD:</u> | <u>TO STANDARD ADD:</u> | <u>TO CONTROL & TESTS ADD:</u> |
|------------------------|-------------------------|------------------------------------|
| 0.1 ml water | 0.1 ml Glucose Standard | 1.0 ml supernatant |
| 0.9 ml 3% TCA Solution | Solution | |
| Stock No. 635-3 | Stock No. 635-100 | |
| | 0.9 ml 3% TCA Solution | |
| | Stock No. 635-3 | |

2. To each tube add:
CAUTION: DO NOT PIPET BY MOUTH
5.0 ml O-Toluidine Reagent, Stock No. 635-6
Mix by lateral shaking.
3. Place all tubes in a vigorously boiling water bath for exactly 10 minutes.
NOTE: If results are calculated from a standard that is assayed with the Test, the boiling period can be 10 ± 1 minute.
4. Quickly remove all tubes and cool to room temperature by placing in tap water for approximately 3 minutes.
5. Transfer contents of tubes to cuvetts and read Absorbance of STANDARD and Tests at 635 ± 15 nm, using BLANK as reference. Complete readings within 30 minutes.

CALCULATIONS:

Use of Standard:

$$\text{Glucose (mg/100 ml)} = \frac{A_{\text{Test}}}{A_{\text{Standard}}} \times 1000*$$

*Represents the concentration of the Glucose Standard (mg/100 ml).

NOTE: If the reading of Test indicates a Glucose concentration greater than 250 mg/100 ml, dilute the test with an equal volume of O-Toluidine Reagent, Stock No. 635-6. Read Absorbance of the diluted Test and multiply result by 2.

DETERMINATION OF LACTIC ACID
REF: Sigma Technical Bulletin No. 826-UV

A. REAGENTS:

1. Lactic dehydrogenase (Sigma stock #826-6).
2. Glycine Buffer (Sigma stock #826-3).
3. NAD preweighed vial (Sigma stock #260-110).
4. Lactic acid standard solution (Sigma stock #826-10).

B. EQUIPMENT:

1. Spectrophotometer

C. DEPROTEINIZATION OF PLATELET SAMPLE:

Trichloroacetic acid extract of sample prepared for glucose assay will be used.

D. ASSAY PROCEDURE:

1. Pipet into each of the NAD vials

2 ml Glycine Buffer
4 ml water
0.1 ml LDH

The number of vials has to be determined based on each vial per 2 assays.

2. Combine the reaction mixture prepared above in a flask.
3. Label an appropriate number of tubes and pipet into each tube; 2.8 ml.
4. To blank add 0.2 ml of 3% TCA. To sample tube add 0.2 ml of respective extract, and mix gently.
5. Incubate at 37°C for 30 min or at 25°C for 45 min.
6. Read absorbance of sample tubes at 340 nm vs. blank as reference. At this point the reading should be stable (less than 0.001 per min increment). If it is changing more than 0.002 per min, an additional incubation for 15 min should be done.

$$A_{340} \times 7.25 \times \frac{10}{3} = \text{mmol L A/L}$$

7. Calculation:

or

$$A_{340} \times 65.1 \times \frac{10}{3} = \text{L A mg/100 ml}$$

Intended Use

The Amersham β -Thromboglobulin (β -TG) RIA Kit provides a quantitative method for the direct measurement of β -TG in human platelet poor plasma over the range 10-225ng β -TG/ml

Summary and Explanation of the Test

The platelet-specific protein, β -Thromboglobulin, is released into the circulation when blood platelets undergo the release reaction.

The platelet release reaction is essential in the primary role of blood platelets in controlling hemostasis. Under normal circumstances, injury to the wall of a blood vessel starts a sequence of events including platelet release, aggregation and formation of a platelet "plug" which stems blood loss and is followed by the formation of a stable fibrin clot. Under certain conditions, these processes can occur within the circulation without injury to the vessel wall.

The appearance of a platelet-specific material in the circulation produced by the platelet release reaction could provide a means of monitoring this reaction.

The presence of a platelet-specific β -globulin had been suggested^{1,2} Subsequently, it was isolated, characterized, and named β -Thromboglobulin (β -TG) by Moore, Pepper and Cash.³ The protein has a molecular weight of 36,000, is thought to comprise six identical sub-units and is probably located in the α -granules of platelets. It is the most abundant platelet-specific protein, though no function has yet been ascribed to β -TG. β -Thromboglobulin is released when platelets undergo their release reaction⁴ and is present in only minute amounts in other tissues.⁵ β -Thromboglobulin can, therefore, be considered as a platelet-specific protein, the release of which is a marker for the platelet release reaction.

Principles of the Procedure

The radioimmunoassay method depends on competition between β -TG and 125 I- β -TG for a limited number of binding sites on a β -TG specific antibody. The amount of 125 I-labeled β -TG bound by the antibody will be inversely proportional to the concentration of unlabeled β -TG present. The antibody-bound 125 I- β -TG is separated by precipitation with ammonium sulfate. After centrifugation and removal of the supernatant, the precipitated radioactivity is measured in a gamma counter. By measuring the proportion of 125 I- β -TG bound in the presence of a series of β -TG standards, the concentration of β -TG in unknown samples can be interpolated from a standard dose response curve.

| | | |
|--|---|-----------------|
| Amersham Corporation 2636 South Clearbrook Drive Arlington Heights, Illinois 60005 1 (800) 323-0668/(312) 364 7523 | In Canada 505 Incoquit Shore Road Oakville, Ontario L6H 2R3 1 (800) 268 5061/(416) 842 2720 | Amersham |
|--|---|-----------------|

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C. Procedural Notes

- Solvents should be prepared fresh on the day of use and stored in glass containers.
 - Recoveries of [^3H]-TXB₂ have routinely been >90% using this method and no [^3H]-recovery marker is necessary.
 - Although this method results in considerably cleaner eluates than conventional methods, a small method blank (>90% B/Bo) may be obtained. This must be accounted for by preparing for each assay, by the same procedure as described above, an extract of distilled water. This should be utilized in one of two ways:
 - Run the extract along with other samples in assay. Extrapolate the apparent TXB₂ value (pg added) using log-logit graph paper and subtract that from each sample value (pg added) before subsequent final calculations to pg/ml.
 - Alternatively, add an aliquot of the distilled water extract to the entire standard curve. Keep the reaction volume constant throughout the assay by adding an equal aliquot of assay buffer to each sample tube. Using this method, any method blank will automatically be corrected for when reading sample values off this standard curve. Assay blinding (Bo) will be somewhat decreased because of the larger sample volume.
- Failure to correct for any detectable method blank obtained (<95% B/Bo), which may vary slightly from day to day, may result in the over-estimation of samples having very low TXB₂ levels.
- Concentration of samples 2-4 times should be adequate for most samples tested. This can be accomplished by using 2-4ml of plasma for the procedure and reconstituting the final extract to 1.0ml.
 - Care must be taken to ensure complete reconstitution of the dried extract by adequate vortexing, since the final volume is less than the original solvent volume of the eluate.
 - This procedure has been validated for single use of BOND-ELUT columns only.

III. URINE

A. Preparation and Extraction

- Acidify an aliquot of the urine to pH 3-3.5 with 2N HCl or 2N Citric Acid.
- Extract with two volumes of ethyl acetate, vortexing 30 seconds.
- Centrifuge 5 minutes at 1000 x g to separate phases.
- Carefully remove 1ml of the organic (upper) phase and elute through a BOND-ELUT SI column prepped with 5.0ml Benzene: Ethyl Acetate (80:20).
- Continue with SI column extraction as described for plasma (Steps 2e-2h).

B. Procedural Notes

- The concentration of TXB₂ in urine is generally high enough to make sample concentration unnecessary. Extraction of 2ml of urine by the procedure above with final reconstitution to 1.0ml results in a final 1:2 sample dilution for assay and should fall within a readable portion of the standard curve for the majority of samples assayed.

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- Recovery of added [^3H]-TXB₂ has been consistently >90% and it is considered unnecessary to employ a [^3H]-TXB₂ recovery marker.
- It is good practice to assay, in each run, an aliquot of extracted distilled water to ensure that non-specific interfering materials have not been introduced from solvents, etc. Any detectable method blank must be corrected for as described for plasma (Section II C).
- It is, of course, the user's responsibility to validate any sample preparation procedure used for urine other than the one recommended here.

APPENDIX II

In the event the user employs a plasma sample preparation procedure other than that recommended in Section V B and Appendix I, he should determine: (1) the recovery of [^3H]-TXB₂ through his procedure, and (2) the effect of his solvents and sample handling techniques with regard to the introduction of non-specific interfering materials. Suggested methods for accomplishing these studies are as follows:

A. [^3H]-TXB₂ Recovery

- Add ~2000cpm [^3H]-TXB₂ per ml of plasma sample.
- Process samples by your selected method with final reconstitution prior to assay being made with assay buffer provided in the kit.
- After allowing at least 30 minutes for complete dissolution, the reconstituted extract should be aliquoted for the RIA and for counting of the [^3H]-TXB₂ recovered.

(Note: Contributions of recovery marker to mass of TXB₂ measured in RIA must be subtracted if the user chooses to add [^3H]-TXB₂ to samples.)

B. Matrix Correction

- Extract, reconstitute, and assay a prostaglandin-free human plasma sample by the same method as planned for your unknowns. Prostaglandin-free human plasma can be made in the following manner: Collect a sufficient volume of normal human plasma following the procedures and precautions given in Part V. Strip the sample overnight with 100mg/ml activated charcoal*. Centrifuge to remove the bulk of the charcoal, then filter down to 0.22µm to remove fine particulates.
- If this results in <95% B/Bo, it will undoubtedly be necessary to correct for this interference by one of the two methods given in #3 of the procedural notes in Appendix I.

*Norit A, the trade name of a decolorizing carbon which is commercially available from Amend Drug and Chemical Co., Irvington, NJ 07111, is recommended.

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APPENDIX I

Procedure for the Extraction of Thromboxane B₂ from Plasma and Urine Using BOND-ELUT Columns

Although the following protocol has been found to be optimal in our laboratory, it must remain the responsibility of the user to assure validation under his conditions. The procedure has been used for the extraction of TXB₂ from plasma and urine and may be applicable to tissue samples after homogenization and centrifugation. All validation data shown in the text utilized the procedures detailed below.

Note: Read this procedure completely before beginning.

I. Equipment and Reagents Required

- BOND-ELUT C₁₈ Extraction Columns, 100 or 200mg, (Analytichem International, Harbor City, CA, Cat. No. 607101 or 607203)
- BOND-ELUT SI Extraction Columns, 500mg, (Analytichem International, Cat. No. 601303)
- Adaptors (Analytichem International, Cat. No. 636)
- VAC-ELUT (Analytichem International), optional
- Millex-GS 0.22µm Sterilizing Filter Units (Millipore Corporation, Bedford, MA, Cat. No. SLGS0250S)
- 2N Hydrochloric or Citric Acid
- Distilled Water
- Methanol, ACS Certified
- Petroleum Ether, ACS Certified
- Ethyl Acetate, ACS Certified
- Benzene, ACS Certified
- Glass Reagent Bottles for Solvent Preparation
- Siliconized Glass or Polypropylene Tubes or Vials for Collection of Extract
- Dry-Down Apparatus and Nitrogen

II. PLASMA

A. Plasma Preparation

1. Acidify the plasma to pH 3-3.5 with 50µl/ml 2N HCl or 100µl/ml 2N Citric Acid. Check pH with pH paper or meter.
2. Centrifuge acidified plasma 10 minutes at 1000 x g at 4°C to separate any proteins that may have denatured and come out of solution as a result of the acidification. (Note: An excess of precipitated proteins may clog the solid-phase C₁₈ column if not removed.)
3. Record the total amount of acid added to the plasma. The dilution effect of acidification must be accounted for in final calculations.

B. Extraction

Step 1 - C₁₈ Column Extraction

- a. Prep the C₁₈ column by successively washing with 2ml MeOH, then 2ml distilled water, pH 3.5. These and all subsequent elutions should be performed under vacuum or under slight positive air pressure in which the flow rate is not more than 1ml/minute.
- b. Apply the prepared plasma sample and pass through the column as described above.
- c. Wash sequentially with 2ml distilled water, pH 3.5, 2ml 15% MeOH, and 2ml Petroleum Ether.

Step 2 - SI Column Extraction

The TXB₂ is eluted off the C₁₈ column from Step 1 directly onto a prepped SI column. Elution with solvent mixtures of increasing polarity will separate the TXB₂ from other more or less polar interferences.

- | | | |
|-----------|---------------------------|------------|
| Solvents: | I. Benzene: EtOAc | - 80:20 |
| | II. Benzene: EtOAc | - 60:40 |
| | III. Benzene: EtOAc: MeOH | - 60:40:2 |
| | IV. Benzene: EtOAc: MeOH | - 60:40:10 |
| | V. Benzene: EtOAc: MeOH | - 60:40:30 |

- a. Prep the SI column with 5ml Solvent I.
- b. Piggy-back the C₁₈ column from Step 1 onto the washed SI column, using the adaptor.
- c. Elute the TXB₂ off the C₁₈ column with 1.0ml EtOAc directly onto and through the SI column. Be sure to force all of the EtOAc through the C₁₈ to avoid loss of recovery.
- d. Remove the C₁₈ column and adaptor and completely wash the EtOAc through the Silica column. (Discard)
- e. Wash the SI column sequentially with 1.0ml Solvent I, 1.0ml Solvent II, 1.0ml Solvent III, and 1.0ml Solvent IV. (Discard)
- f. Elute the TXB₂ off the SI column with 3.0ml Solvent V into a polypropylene or siliconized glass tube or vial.
- g. Dry the extract under N₂ and reconstitute to desired volume with assay buffer. Mix and allow to sit for at least 30 minutes.
- h. Filter the extract through a 0.22µm Millex filter using a polypropylene or siliconized glass syringe into a suitable tube or vial.

- B. The thromboxane B₂ standards are prepared in the assay phosphate buffer. The effect of other sample matrices upon the assay system must be determined by the investigator.
- C. Any exogenous radioactivity will lead to erroneous results. Proper controls should be employed to determine if samples are contaminated.

IX. PERFORMANCE CHARACTERISTICS

A. Reproducibility of the Assay

Reproducibility was determined by running multiple duplicate analyses of several spiked plasma samples both within and between assay.

| Sample | Mean pg/ml \pm S.D. (n = 6) | |
|--------|-------------------------------|-----------------|
| | Intra | Inter |
| A | 20.8 \pm 3.2 | 13.5 \pm 2.3 |
| B | 33.2 \pm 3.0 | 40.3 \pm 1.5 |
| C | 56.8 \pm 2.3 | 64.3 \pm 1.5 |
| D | 100.9 \pm 7.7 | 107.8 \pm 6.6 |

B. Accuracy

The accuracy of the method was evaluated by determining the recovery of TXB₂ spiked into human plasma and assayed by the suggested protocol.

| Added pg/ml | Measured pg/ml | Recovered pg/ml | Recovery % |
|----------------|-------------------|--------------------|---------------|
| 0 | 10.7 | | |
| 25 | 35.4 | 24.7 | 99% |
| 50 | 62.6 | 51.9 | 104% |
| 100 | 103.2 | 92.5 | 93% |

C. Linearity

Linearity was evaluated in two ways: (1) by extracting various volumes of plasma and assaying 100 μ l, and (2) by extracting a single volume (2ml) and assaying various aliquots.

| Sample | pg/ml, Normalized | |
|----------|-----------------------------|--------------------------|
| | Plasma Volume Extracted, ml | Aliquot Assayed, μ l |
| Sample A | 12.5 | 25 |
| Sample B | 47.3 | 50 |
| | 14.8 | 100 |
| | 47.8 | 15.5 |
| | 49.5 | 52.8 |
| | 48.1 | 14.8 |
| | | 50.8 |
| | | 47.8 |

D. Comparative Sample Values: [125I]-Kit vs. [3H]-Kits

Sample values were compared by assaying an extracted plasma sample at various dilutions in both the [125I] kit (NEK-025) and the [3H] kit (NEK-007). The sample extract was concentrated four times in order to cover the standard curve range of both kits.

| Extract Dilution | pg/ml Extract | |
|------------------|---------------|-----------|
| | [125I]-Kit | [3H]-Kit |
| straight | off curve | 1656 |
| 1:2 | off curve | 997 |
| 1:4 | 599 | 466 |
| 1:8 | 274 | 269 |
| 1:16 | 122 | 135 |
| 1:32 | 57 | 89 |
| 1:64 | 22 | off curve |
| 1:128 | 13 | off curve |

E. Sensitivity

Defined as the mass corresponding to twice the standard deviation of the zero binding, the sensitivity of the system was found to be approximately 0.8pg added.

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Table 2 - Sample Calculations

| | Tube No. | CPM | Average CPM | Net CPM | % B/Bo | Sample Value |
|--------------|----------|-------|-------------|---------|--------|--------------|
| Total Counts | 1 | 16210 | 16027 | | | |
| | 2 | 15844 | | | | |
| Blank | 3 | 356 | 376 | | | |
| | 4 | 396 | | | | |
| "0" Standard | 5 | 8888 | 8864 | 8488 | 100 | |
| | 6 | 8940 | | | | |
| 1pg | 7 | 8128 | 8131 | 7755 | 91.4 | |
| | 8 | 8134 | | | | |
| 2.5pg | 9 | 7578 | 7540 | 7164 | 84.4 | |
| | 10 | 7502 | | | | |
| 5pg | 11 | 6778 | 6755 | 6379 | 75.2 | |
| | 12 | 6732 | | | | |
| 10pg | 13 | 5222 | 5410 | 5034 | 59.3 | |
| | 14 | 5598 | | | | |
| 25pg | 15 | 3640 | 3686 | 3310 | 39.0 | |
| | 16 | 3732 | | | | |
| 50pg | 17 | 2246 | 2366 | 1990 | 23.4 | |
| | 18 | 2486 | | | | |
| 100pg | 19 | 1448 | 1547 | 1171 | 13.8 | |
| | 20 | 1646 | | | | |
| Sample | 21 | 7150 | 7193 | 6817 | 80.3 | 3.4pg |
| | 22 | 7235 | | | | |

VIII. LIMITATIONS

A. The following compounds have been checked for cross-reactivity. The percentages are calculated at the 50% B/Bo point.

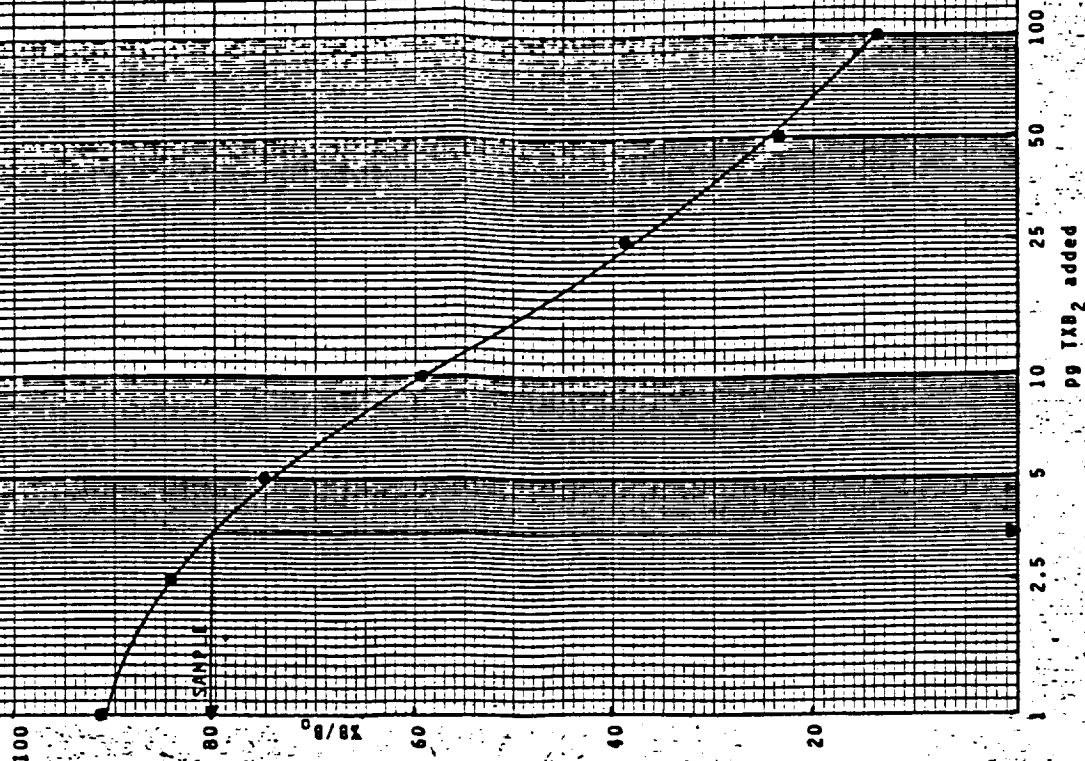
| Compound | % Cross-Reactivity | Cross-Reactivities Compound | % Cross-Reactivity |
|---------------------|--------------------|-----------------------------|--------------------|
| TXB ₂ | 100 | Arachidonic Acid | 0.005 |
| PGD ₂ | 3.9 | DHAPGE ₂ | 0.003 |
| PGE ₂ | 0.23 | PGA ₁ | 0.002 |
| PGF _{2α} | 0.07 | PGB ₁ | <0.001 |
| PGE ₁ | 0.06 | DHAPGF _{2α} | <0.001 |
| 6KPGF _{1α} | 0.06 | Linoleic Acid | <0.001 |
| PGF _{1α} | 0.02 | Oleic Acid | <<0.001 |
| PGA ₂ | 0.007 | Palmitic Acid | <<0.001 |

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TYPICAL STANDARD CURVE

DO NOT USE TO CALCULATE SAMPLES



9. Pipet 100µl of tracer solution into each tube and mix.
10. Pipet 100µl of antiserum into all tubes beginning with tube 5 and vortex thoroughly for 2-5 seconds.
11. Incubate overnight (16-24 hours) at 2-8°C.
12. At the end of the overnight incubation, place all tubes in an ice bath.
13. Pipet 1ml of cold precipitating reagent into all tubes beginning with tube 3. Vortex each tube thoroughly for 2-5 seconds.
14. Allow tubes to incubate at 2-8°C for 20-30 minutes.
15. Centrifuge the tubes in a refrigerated centrifuge at 1000-2000 x g for 30 minutes.
16. Decant the supernatants of all tubes beginning with tube 3 and allow to drain for ~ one minute on absorbent paper. Blot the tubes to remove residual liquid at the rim.
17. Count all tubes in a gamma counter. At normal efficiencies, a one minute count time is sufficient.
18. Calculate results as described in Section VIII.

Table 1 - Thromboxane B₂ Assay Protocol Schematic
(All volumes are in microliters)

| | Tube No. | Buffer | Standard | Samples | Tracer | Antibody |
|--------------|------------|--------|----------|---------|--------|----------|
| Total Counts | 1-2 | -- | -- | -- | 100 | -- |
| Blank | 3-4 | 200 | -- | -- | 100 | -- |
| "0" Standard | 5-6 | 100 | -- | -- | 100 | 100 |
| Standards | 7-20 | -- | 100 | -- | 100 | 100 |
| Samples | 21,22 etc. | -- | -- | 100 | 100 | 100 |

Incubate overnight (16-24 hours) at 2-8°C. Add 1ml of cold precipitating reagent to all tubes except total counts. Mix, incubate for 20-30 minutes at 2-8°C, and centrifuge at refrigerated temperatures for 30 minutes at 1000-2000 x g. Decant all tubes except total count tubes, blot and count.

D. Precautions

1. Pipetting must be done reproducibly and accurately. Thromboxane B₂ has the tendency to adhere to many surfaces. To minimize assay interference from this "sticking" problem, use only polypropylene or siliconized glass pipets, pipet tips and tubes when transferring diluted materials or incubating.
2. An aliquot of an appropriate control should be assayed to account for any method blank that may arise as a result of the extraction or chromatographic steps (see Appendix 1). It is the user's responsibility to check and correct for non-specific matrix and solvent effects.
3. Inadequate incubation time with the precipitating reagent or centrifugation time or speed may result in incomplete precipitation of bound counts.
4. Inadequate centrifugation speed or prolonged inversion after decanting may cause the pellets to become dislodged from the bottom of the tubes.
5. Since the assay is sensitive to sub-picogram quantities of TXB₂, extreme care must be taken to avoid contamination of laboratory equipment (such as pipettors) with TXB₂.

VII. PROCEDURE FOR CALCULATING UNKNOWN

After counting has been completed, the concentration of thromboxane B₂ in the samples is determined from a standard curve. The following method is suggested. (See Table 2 for sample calculations.)

1. If all tubes have been counted for the same period of time, use the total accumulated counts; otherwise, correct all raw counts to counts per minute (CPM).
2. Average the counts for each set of duplicates.
3. Calculate the average NET counts for all standards and samples by subtracting from each the average blank counts (tubes 3-4).
4. Determine the normalized percent bound ($\% B/Bo$) for each standard and sample as follows:

$$\% B/Bo = \frac{\text{Net cpm of standard or sample}}{\text{Net cpm of } ^{125}\text{I standard}} \times 100$$

5. Using semi-logarithmic graph paper, plot $\% B/Bo$ for each standard versus the corresponding amounts of thromboxane B₂ added in picograms (pg). See Figure 3 for a typical standard curve using the standard protocol.)
6. Determine the pg thromboxane B₂ in each sample by interpolation from the standard curve. Since the standard curve is expressed as pg thromboxane B₂ added, sample values must then be corrected for method blank, aliquots, dilution and recovery to determine the original concentration in the sample.

NOTE: Any samples with concentrations which are above the range of the standard curve may be diluted with assay buffer and re-assayed. The values obtained are then multiplied by the appropriate dilution factor.

The recommended procedure is detailed in Appendix I, pp. 14-17 and involves three major steps in the purification of samples prior to assay:

1. Extraction, concentration, and partial purification of TXB₂ from plasma into organic solvent using BOND-ELUT C₁₈ columns.
2. Further purification using BOND-ELUT Si columns followed by dry-down of eluate containing TXB₂.
3. Filtration of reconstituted eluate to remove interfering particulate material apparently resulting from solvent/column interaction.

2. Urine

TXB₂ probably of renal origin is present in the urine in addition to a number of metabolites derived from thromboxane in the peripheral circulation (32). Extraction by conventional methods and subsequent purification on BOND-ELUT Si columns (see Appendix I, pp. 14-17) has yielded linear results and quantitative recovery of added TXB₂.

3. Tissues

Tissues must be homogenized prior to extraction and assay. Methods for accomplishing this have been reported (25,28). It is suggested that the procedure outlined for plasma (see Appendix I, pp. 14-17) be followed after the tissue is homogenized, centrifuged, and the pellet discarded. Our experience with these procedures is limited and it remains the responsibility of each investigator to validate his own system.

It will be essential for the investigator to dilute the final sample for assay in the assay buffer supplied with the kit in order to ensure adequate precipitation by the Precipitating Reagent.

VI. ASSAY

A. Equipment and Reagents Required

In addition to the reagents supplied with the kit, the following materials are required for assay:

1. Pipettors and/or pipets that accurately and precisely deliver the required volumes. Pipets and/or pipet tips used to transfer diluted standards, tracer, or samples should be made of polypropylene or siliconized glass. Do not use those made of unsilicized glass. Positive displacement pipettors may be most convenient for dispensing the precipitating reagent**.
2. Siliconized glass test tubes (these can be prepared by treating borosilicate glass tubes with Sigmacote*), or polypropylene test tubes.
3. Test tube rack.
4. Ice bath.
5. Beakers or flasks.
6. Vortex mixer.
7. Centrifuge, refrigerated, with swinging bucket rotor.
8. Gamma counter.

*Sigmacote is the trade name of a siliconizing agent which is commercially available from Sigma Chemical Co., St. Louis, MO 63178.

**Positive displacement pipettors, such as Reipet, Dispensette, and Oxford dispensers are readily available through Fisher and other chemical supply companies.

B. Preparation of Thromboxane B₂ Working Standards

An aliquot of the reconstituted TXB₂ standard concentrate is diluted with assay buffer in order to prepare a series of working thromboxane B₂ standards (tubes b-h in the dilution scheme below). A suggested dilution scheme to cover a standard curve range of 1pg to 100pg added (per 0.1ml) is shown below. Other dilution schemes which cover this range can be used, but the assay buffer provided with the kit must be used for dilution of the standards. The assay buffer contains additives which minimize the non-specific adsorption of thromboxane B₂ to the wall of the test tubes.

NOTE: Working standards must be freshly prepared on the day of use. Working standards should not be stored overnight before use. Use only siliconized glass test tubes, or polypropylene tubes. Use polypropylene or siliconized glass pipets or pipet tips for transferring standard solutions. Do not use pipets of unsilicized glass.

Suggested dilution scheme:

| Tube | Concentration (pg/0.1ml)* |
|------|---|
| a | 0.1ml (100µl) standard + 1.9ml assay buffer 500 |
| b | 0.2ml of dilution a + 0.8ml assay buffer 100 |
| c | 0.4ml of dilution b + 0.4ml assay buffer 50 |
| d | 0.4ml of dilution c + 0.4ml assay buffer 25 |
| e | 0.4ml of dilution d + 0.6ml assay buffer 10 |
| f | 0.4ml of dilution e + 0.4ml assay buffer 5 |
| g | 0.4ml of dilution f + 0.4ml assay buffer 2.5 |
| h | 0.4ml of dilution g + 0.6ml assay buffer 1 |

*This concentration represents actual mass added to assay tube.

Dilutions b through h should be used for the standard curve.

C. Radioimmunoassay Protocol

The protocol below is written for the assay of plasma samples as described in Section V B and Appendix I. If other protocols are used, keep standard and sample tubes identical in terms of volume and as similar as possible in terms of matrix.

Carefully read Sections V and VI D before proceeding with the assay.

1. Prepare all reagents according to directions in Section IV.
2. Equilibrate all reagents to room temperature and mix before use.
3. Label duplicate tubes for total counts, blank, each standard, and each sample.
4. Place tubes in a suitable test tube rack.
5. (Refer to Table 1 for a synopsis of steps.)
6. Pipet 200µl of assay buffer into tubes 3-4 (blank tubes).
7. Pipet 100µl of assay buffer into tubes 5-6 (zero standard tubes).
8. Pipet 100µl of each diluted standard (b through h) into tubes 7-20. Pipet 100µl of each sample extract in duplicate into the appropriate tubes starting with tube 21.

INSTRUCTIONS RELATING TO THE HANDLING, USE, STORAGE, AND DISPOSAL OF THIS RADIOACTIVE MATERIAL.

This radioactive material may be received, acquired, possessed, and used only by research laboratories for in vitro laboratory tests not involving internal or external administration of the material, or the radiation therefrom, to human beings or animals. Its receipt, acquisition, possession, use, and transfer are subject to the regulations and a general license of the U.S. Nuclear Regulatory Commission or of a State with which the Commission has entered into an agreement for the exercise of regulatory authority.

1. All radioactive materials should be stored in specifically designated posted areas.
2. All work with these materials should be carried out only in authorized areas.
3. No pipetting should be done by mouth.
4. There should be no smoking or eating within the work area.
5. Hands should be washed after handling radioactive materials.
6. Any spilled material should be wiped up quickly and thoroughly and the contaminated substances transferred to a suitable receptacle. The surfaces involved should be washed thoroughly with an appropriate decontaminant.
7. When use of the tracer reagent has been completed, empty and decontaminate the vial. This radioactive material can be discarded into the sanitary sewerage system, provided the discharge concentration of 10^{-5} does not exceed 4×10^{-5} $\mu\text{Ci}/\text{ml}$. (See 10 CFR 20.303 for maximum daily, monthly and yearly quantity discharge limits.)
8. Prior to disposal of the empty, uncontaminated kit and tracer containers in unrestricted areas, remove or deface the radioactive material labels or otherwise clearly indicate that the containers no longer contain radioactive material.

B. TXB₂ Antibody

The rabbit anti-TXB₂ is supplied ready to use. Stored at 4°C, the solution is stable for at least two months.

C. TXB₂ Standard Concentrate

To reconstitute, add exactly 1.0ml of distilled water. The reconstituted solution contains 100ng/ml of thromboxane B₂ in buffer. Immediately before use in the assay, dilute an aliquot of the stock solution to prepare standards. A suggested procedure for preparing diluted standards appears in Section VI. Do not store diluted standards. Pipets and/or pipet tips used to transfer diluted standard must be made of polypropylene or siliconized glass. Do not use those made of unsiliconized glass. Store the remaining concentrated standard at 4°C. Under these conditions, the solution is stable for at least two months.

D. Assay Buffer

The solution consists of 0.9% NaCl, 0.01M EDTA, 0.3% bovine γ -globulin, 0.005% Triton-X-100 and 0.05% sodium azide in 50mM phosphate buffer, pH 6.8. When stored at 4°C, it is stable for at least two months.

E. Precipitating Reagent

The solution contains 16% polyethylene glycol (PEG 6000) and 0.05% sodium azide in 50mM phosphate buffer, pH 6.8. Stored at 4°C, it is stable for at least two months. This reagent is quite viscous and use of a positive displacement device facilitates dispensing.

V. SAMPLE HANDLING

A. Collection and Storage

It is recommended that all samples be processed immediately after collection and assayed as soon as possible.

Blood samples should be collected in pre-chilled siliconized glass or polypropylene test tubes coated with a solution of 4.5mM EDTA combining a prostaglandin synthetase inhibitor such as indomethacin or aspirin (24). Indomethacin has been reported to be very effective at concentrations of up to 10 $\mu\text{g}/\text{ml}$ and we have been able to demonstrate the lack of interference in the assay at these concentrations. The plasma fraction should be isolated from the whole blood as soon as possible after collection and frozen at -70°C if it is not assayed on same day.

Tissue samples should be deep frozen on dry ice or in liquid nitrogen immediately after collection. If tissues are not analyzed immediately, samples should be stored at -70°C or lower. Tissue samples should be processed in the presence of prostaglandin synthetase inhibitors (25), such as indomethacin, at the concentration cited above.

B. Preparation for Assay

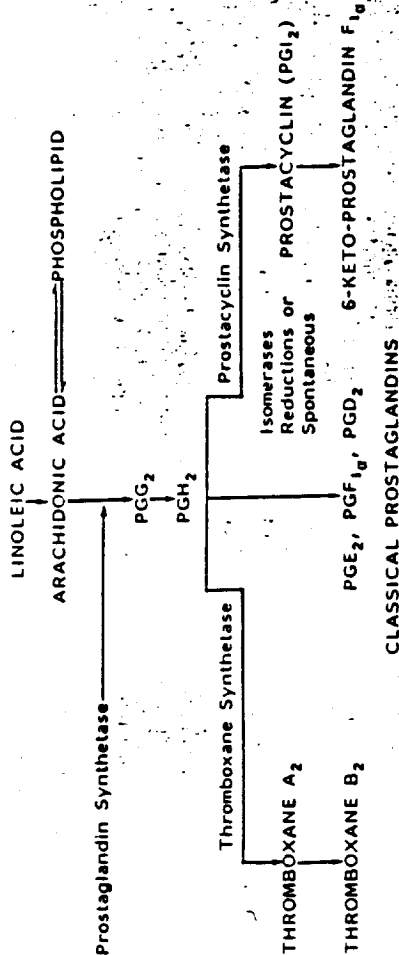
1. Plasma

In many plasma samples, the low level of analyte will necessitate extraction and subsequent concentration prior to assay. Normal plasma TXB₂ levels as low as 10-40pgs/ml have been reported (26,27). There is considerable ambiguity in the literature concerning the extraction of prostaglandins. Useful solvent extraction procedures have been reported (25,28), but considerable care must be taken to produce a sample clean enough for assay. At NEN, solid-phase extraction using modifications of procedures reported by Skrinška and Lucas (29) has been found preferable to liquid-liquid extraction methods. Commercially available chemically bonded extractants have been used by others (30,31). These have yielded consistently higher recoveries and considerably cleaner eluates than other extraction methods in our hands.

*BOND-ELUT C₁₈ and SI extraction columns are available from Analytichem International, Harbor City, CA. SEP-PAK C₁₈ and SI extraction columns are available from Waters Associates, Milford, MA.

Figure 1

Schematic of Arachidonic Acid Metabolic Pathway



II. EXPLANATION OF THE TEST

Thromboxane B₂ (TXB₂) has been detected in plasma and urine as well as in a variety of human tissues; e.g. lung and kidney (3). The level of TXB₂, however, is often very low and sensitive procedures are required for accurate measurement. The original methods, bioassay and GC/mass spectrometry (18,19), were either too insensitive, non-specific, or cumbersome for routine use. The development of specific antisera resulted in radioimmunoassays (20,21) which started to approach the sensitivity required. However, the relatively low specific activity of the tritiated TXB₂ used in these assays limited the sensitivity that could be obtained. It is only recently that high specific activity, iodinated, prostaglandin tracers have been developed and used to increase the sensitivity of these RIA systems (22,23).

The New England Nuclear Thromboxane B₂ Radioimmunoassay Kit is based on the use of an iodinated analog of Thromboxane B₂ as tracer and rabbit anti-thromboxane B₂ as the antiserum (specific antibody).

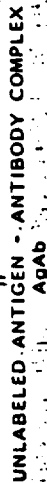
III. PRINCIPLE OF THE METHOD

The basic principle of this radioimmunoassay is competitive binding, where a radioactive antigen competes with a non-radioactive antigen for a fixed number of antibody binding sites. When unlabeled antigen from standards or samples and a fixed amount of tracer (labeled antigen) are allowed to react with a constant and limiting amount of antibody, decreasing amounts of tracer are bound to the antibody as the amount of unlabeled antigen is increased.

In the New England Nuclear Thromboxane B₂ [¹²⁵I] RIA Kit, separation of the antibody-antigen complexes from free antigen is achieved by precipitation of the antibody-bound tracer with polyethylene glycol in the presence of carrier immunoglobulin. After centrifugation, the supernatant, containing the unbound antigen, is decanted and the pellet, containing the antibody-antigen complex, is counted in a gamma counter. Results obtained for the standards are used to construct a standard (dose-response) curve from which the unknowns are read by interpolation.



Unlabeled Antigen (In standard solutions or unknown samples)



IV. REAGENT DESCRIPTION AND PREPARATION

This kit is intended for research use and not for diagnostic purposes. All necessary reagents are supplied for 125 (NEK-024) or 250 (NEK-024A) assay tubes when the suggested assay protocol is followed.

| NEK-024 | NEK-024A | Kit Components |
|-----------|-----------|---|
| 125 tubes | 250 tubes | |
| 1 vial | 2 vials | TXB ₂ Antibody, 13ml |
| 1 vial | 2 vials | TXB ₂ [¹²⁵ I] Tracer Concentrate, 0.75ml |
| 1 vial | 2 vials | TXB ₂ Standard Concentrate, lyophilized |
| 1 bottle | 2 bottles | Assay Buffer, 125ml |
| 1 bottle | 2 bottles | Precipitating Reagent, 125ml |

The Thromboxane B₂ Kit is shipped at ambient temperature and should be stored at 4°C upon receipt. Stability of the individual kit components and precautions for handling are described below.

A. TXB₂ [¹²⁵I] Tracer

The vial of tracer concentrate contains approximately 2μCi of TXB₂ [¹²⁵I] in 0.75ml of organic solvent. Stored at 4°C, the tracer concentrate is stable for at least one month from date of receipt.

For use in assay, an appropriate aliquot of the tracer concentrate is diluted 1:20 (v:v) in assay buffer in a siliconized glass or polypropylene tube or vial. (For example, for a 20 tube assay, dilute 0.1ml of tracer concentrate with 2.0ml assay buffer.) Dilute only enough tracer for use in each assay. Do not store and reuse. Return the unused portion of tracer concentrate to 4°C storage.

Pipets and/or pipet tips used to transfer the tracer solution must be of polypropylene or siliconized glass. Do not use those made of unsiliconized glass.

1. INTRODUCTION

The schematic for the "in vivo" metabolic pathways of the major prostaglandins, shown in Figure 1, represents only the most general outline of a very complex series of biochemical interconversions. Linoleic acid is an essential fatty acid which is metabolized to arachidonic acid, the starting compound of the cascade. Arachidonic acid is stored in cell walls esterified in phospholipids (1). Upon demand, arachidonic acid may be released from the cell wall by phospholipase A₂ (2).

Prostaglandin synthetase contains both cyclo-oxygenase and peroxidase activities to convert arachidonic acid to prostaglandin endoperoxide. Cyclo-oxygenase enzymatically metabolizes arachidonic acid to prostaglandin G₂ (PGG₂), a cyclic endoperoxide, while peroxidase reduces PGG₂ to another cyclic endoperoxide, prostaglandin H₂ (PGH₂) (3,4). Both PGG₂ and PGH₂ have a half-life of about 5 minutes at 37°C in aqueous buffer at pH 7.4 (5). Prostaglandin endoperoxide PGH₂ is considered a pivotal compound, since it is metabolized by three different reaction pathways:

1. In the presence of thromboxane synthetase, known to be present in large amounts in platelets (4,6), PGH₂ is converted to thromboxane A₂. Thromboxane A₂ is rapidly hydrolyzed to thromboxane B₂ (TXB₂).
2. Prostacyclin synthetase, which has been demonstrated in the microsomal fraction of endothelial cells (7,8), hydrolyzes PGH₂ to prostacyclin. Prostacyclin is unstable and converts to 6-keto-prostaglandin F_{1α} (6-keto-PGF_{1α}).
3. Classical prostaglandins, PGE₂, PGF_{2α}, can be formed from the prostaglandin endoperoxide PGH₂ either spontaneously or enzymatically in the absence of either thromboxane or prostacyclin synthetases (5). In addition, the classical prostaglandins may be formed in association with the other two pathways.

Because of the opposing effects of prostacyclin and thromboxane A₂ synthesis, there is the potential for the delicate control of hemostasis and arterial thrombosis in vivo. That is, thromboxane A₂ promotes platelet aggregation and is a vasoconstrictor, while prostacyclin prevents platelet aggregation and is a vasodilator. By quantitatively measuring both stable conversion products - TXB₂ and 6-keto-PGF_{1α} - researchers can study normal as well as pathological mechanisms. For example, pathological states can be generated in the experimental model by manipulating the activity of selected enzyme(s) (8-11). Pharmacological agents can then be assessed for their effectiveness in preventing or alleviating various pathological conditions. Hemorrhagic and thromboembolic disorders under investigation include thromboembolism (8), atheromatous plaques (12) diabetes (13) and a variety of congenital and acquired bleeding disorders which include platelet storage pool disorders and secretory defects (14-16). Certain organs such as the lung and kidney and possibly certain cells in tissue culture can produce both thromboxane A₂ and prostacyclin (1,4,8). In addition, there appears to be a species dependent predisposition for producing larger quantities of one of the two compounds (6,17). In order to fully understand the biological roles of thromboxane A₂ and prostacyclin, it is first necessary to be able to monitor their production. For thromboxane A₂ and prostacyclin, their respective stable conversion products (TXB₂ and 6-keto-PGF_{1α}) are the parameters of choice.



NEN New England Nuclear®

NEK-024 NEK-024A
125 tubes 250 tubes

| | |
|----------|-----------|
| 1 vial | 2 vials |
| 1 vial | 2 vials |
| 1 vial | 2 vials |
| 1 bottle | 2 bottles |
| 1 bottle | 2 bottles |

Kit Components

TXB₂ Antibody, 13ml
TXB₂ [¹²⁵I] Tracer Concentrate, 0.75ml
TXB₂ Standard Concentrate, lyophilized
Assay Buffer, 125ml
Precipitating Reagent, 125ml

Thromboxane B₂ [¹²⁵I] RIA Kit

Catalog No.

NEK-024 NEK-024A
(125 tubes) (250 tubes)

Instruction Manual
for the Measurement of
Thromboxane B₂ Levels
in Tissue and Biological Fluids.

CAUTION, NOT FOR USE IN HUMANS OR CLINICAL DIAGNOSIS: These products are intended for research or manufacturing use only. They are pharmaceutically unrefined, and verification of their suitability for use in humans or as clinical diagnostic reagents and the compliance with all Federal and State laws regulating such applications are solely the responsibility of the purchaser.

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LIMITED WARRANTY
New England Nuclear (NEN) warrants that, at the time of shipment, the NEN product is free from defects in material and workmanship and conforms to the specifications set forth in the NEN product literature. NEN makes no other warranty, express or implied, and the user assumes all responsibility for the use of the product. NEN's liability is limited to the replacement of the product, or a refund of the purchase price, at NEN's option. No other damages, including consequential damages, shall be recoverable. This warranty is void where prohibited by law. NEN's liability is limited to the replacement of the product, or a refund of the purchase price, at NEN's option. No other damages, including consequential damages, shall be recoverable. This warranty is void where prohibited by law.

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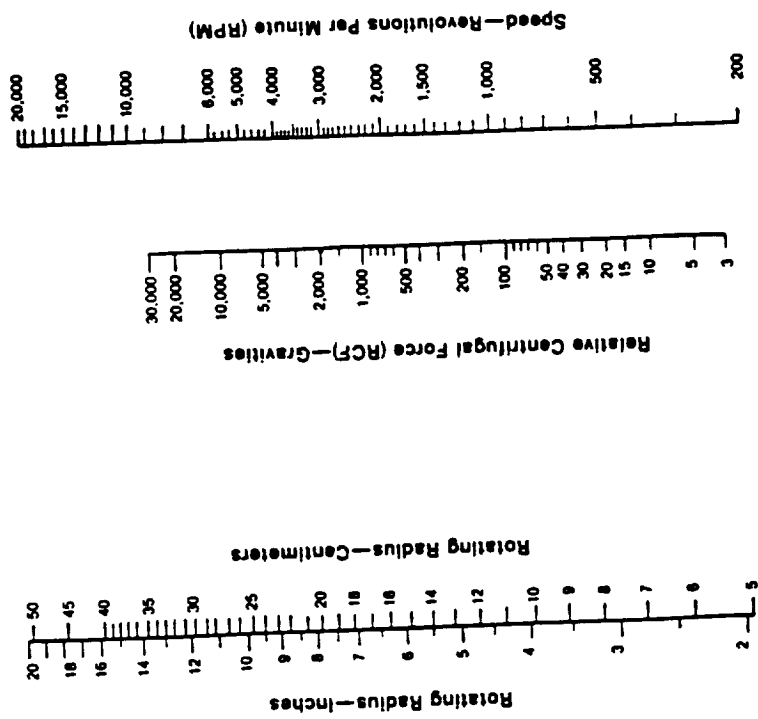


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Nomograph for Relating Relative
Centrifugal Force (RCF) to Revolutions Per Minute (RPM)



1. Measure the rotating radius of centrifuge.
2. Determine recommended RCF for the product ($> 1500g$).
3. Using a straight edge, connect rotating radius with recommended RCF.
4. Correct RPM setting is the point where straight edge intersects the RPM scale.

Max. Physiological

| Compound | Dose | Dose Tested | Effect |
|-------------------|------------|-------------|---|
| Pontopon® — Roche | 53 µg/ml | 100 µg/ml | Lowers β -TG level by 3.5 ng/ml |
| Aspirin | 1.2 mg/ml | 10 mg/ml | Lowers β -TG level by 2 ng/ml |
| Heparin | 0-30 IU/ml | 10 IU/ml | Lowers β -TG level by 1-2.5 ng/ml |
| | | 50 IU/ml | Lowers β -TG level by 14-15 ng/ml |

Sensitivity

The lowest detectable level of β -TG that can be distinguished from zero with 95% confidence (± 2 S.D.) is 5 ng/ml.

Accuracy

The mean % recovery of β -TG added to 25 clinical samples was 102%.

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 - B) Ludlam, C.A., Bolton, A.E., Moore, S. and Cash, J.D., New rapid method for diagnosis of deep venous thrombosis, *Lancet*, 2, 259-260 (1975).
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interest should be verified in the user's laboratory. It should be noted that elevated β -TG levels may result from improper sample collection and processing.

Performance Characteristics

Reproducibility

The reproducibility of the β -TG RIA Kit has been evaluated and the results obtained in a hospital laboratory are shown in Table 2.

Table 2. Reproducibility of the β -TG RIA Kit

| | Controls | | |
|---|----------|------|-------|
| | A | B | C |
| Number of assays* | 39 | 54 | 39 |
| Mean ng β -TG/ml | 19.6 | 43.6 | 108.0 |
| Standard Deviation (each result the mean of duplicates) | 1.9 | 4.6 | 16.5 |
| Coefficient of variation % | 9.9 | 10.7 | 15.3 |

*4 operators, 14 kit batches aged from 2-14 weeks over a 9-month period

Specificity

The following compounds were tested and were found not to interfere with the assay in concentrations up to those calculated to be the maximum possible circulating level.

| Compound | Max. Physiological Dose | Dose Tested |
|---|-------------------------|----------------|
| Pethidine | 0.5mg/ml | 10mg/ml |
| Morphine | 13 μ g/ml | 500 μ g/ml |
| Septa [®] - B Wellcome | 1mg/ml | 0.4mg/ml† |
| Dextran | 17mg/ml | 20mg/ml |
| Platelet-factor 4 | — | 0.4mg/ml |
| Ethylene-diamine-tetra-acetic acid (EDTA) | 3mg/ml | 20mg/ml |
| Theophylline | — | 2.56mg/ml |
| Warfarin | 0.1mg/ml | 100mg/ml |

†not soluble in higher concentrations

The interference levels recorded by Heparin, Pontopon[®] and aspirin are in excess of those normally observed in patients, therefore, unlikely to cause problems in measurement of patient samples.

11

| Sample Calculation Tube No. | Standard | Counts/60 sec. | ng β -TG/ml |
|-----------------------------|-----------|----------------|-------------------|
| 1 | 10ng/ml | 12082 | |
| 2 | " | 11862 | |
| 3 | 21ng/ml | 10339 | |
| 4 | " | 10209 | |
| 5 | 54ng/ml | 7657 | |
| 6 | " | 7732 | |
| 7 | 101ng/ml | 5880 | |
| 8 | " | 5829 | |
| 9 | 218ng/ml | 3739 | |
| 10 | " | 3900 | |
| 11 | Unknown 1 | 10543 | 20 |
| 12 | " | 10356 | |
| 13 | Unknown 2 | 8302 | 44 |
| 14 | " | 8103 | |
| 15 | Unknown 3 | 5323 | 119 |
| 16 | " | 5392 | |

Details of Calibration

The standards supplied with the β -TG RIA Kit have been accurately calibrated against primary β -TG standards at Amersham International plc, and the exact value of each standard is printed on the vial in ng β -TG/ml.

Limitations

Care should be taken to ensure that the patient samples do not contain radioactivity sufficient to cause interference with the assay (See Interfering Substances — Sample Collection).

EXPECTED VALUES

It is postulated that the measurement of β -TG levels may be useful in assessing platelet release, which is a characteristic of numerous disorders. However, current studies have not yet clearly defined a clinical use. Therefore, this kit is intended for research use only and the results obtained should not be used for clinical purposes.

For informative purposes, the mean values for β -TG levels were within the range 24-28ng β -TG/ml on 124 β -TG determinations on 31 apparently normal individuals obtained at 4 centers. Ninety-five percent of the values were below 52ng β -TG/ml. This does not imply that all normal persons will have values in this range or that persons with platelet disorders will fall outside this range. For research applications, the values to be found in controls and populations of

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5. Decantation

The supernatants should be decanted within 10 minutes of the end of centrifugation. After decantation, the tubes must be drained for at least five minutes. It is important to ensure that the tubes are not placed upright once they have been inverted until draining is complete.

6. Radioactivity Counting

The following points should be noted in the radioactivity counting:

- Sufficient counts should be accumulated to reduce the statistical variation to within acceptable limits — for example, the standard deviation on 10,000 counts is 1%.
- The use of glass containers for secondary containment of the assay tubes in the gamma counter should be avoided, since the attenuation of the low energy ^{125}I gamma radiation by glass is relatively high. Not only is the count rate markedly reduced but serious errors may arise due to variations in the wall thickness of the glass container. Where secondary containment is required, molded plastic tubes or vials are preferred.
- Possible variations in the count rates due to the positioning of the assay tube in the gamma counter should also be checked.

Quality Control

The user may apply the following checks on assay performance:

- The time required to accumulate 10,000 counts in the 10ng/ml standard (tubes 1 and 2) should be not more than 1-4 minutes depending on the efficiency of the counter and the age of the kit.
- The shape of the standard curve should be similar to that of the example shown in Figure 1.
Mean counts in tubes 1, 2, should be ≥ 1.7
The ratio: Mean counts in tubes 7, 8
- (Background counts should be subtracted in calculating this ratio)

Assay Modification

In accordance with good laboratory procedure, at least one control sample of human platelet poor plasma should be included in each assay run.

Blanks

Corrections for samples containing radioactivity can be made by assaying the sample and a blank tube for the sample.

The original sample is assayed according to the usual protocol. Blanks are saved according to the sample procedure substituting 200 μL of water for the antiserum solution. In addition, a blank must be run on a standard (preferably the 10ng/ml standard) to use this correction. If the counts for the standard blank and the blank for the patient sample are within a small range (5%), then the radioactivity in the sample does not interfere with the assay because the radioactive material is not precipitated by ammonium sulfate and is decanted in the supernatant. If the counts for the patient sample blank are higher than that for the standard, the radioactivity present can interfere.

A corrected standard curve should be drawn plotting the standard counts — the

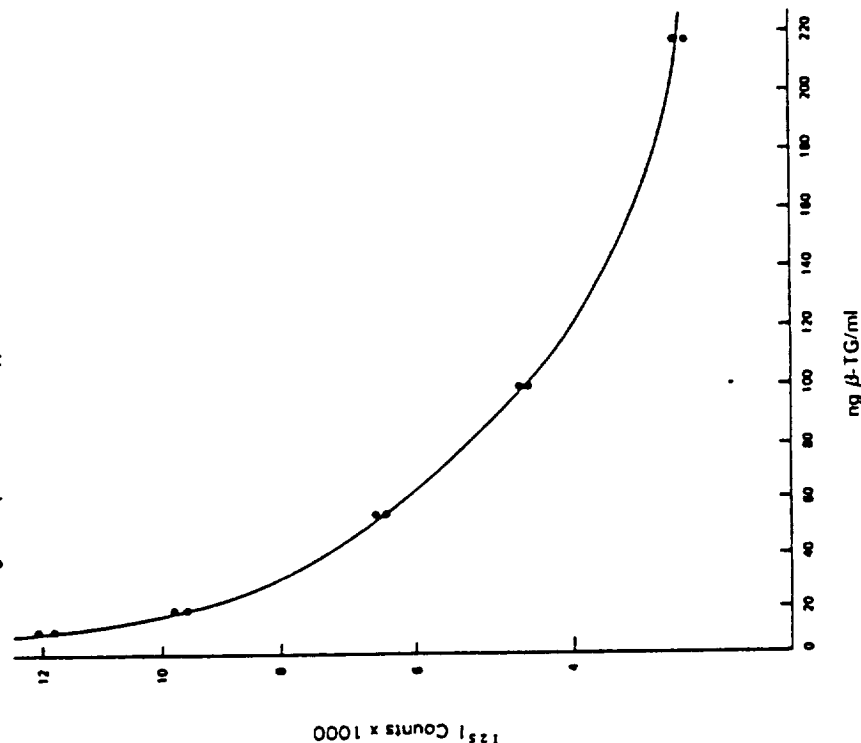
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standard blank counts versus β -TG concentration and the sample blank counts — the concentration determined using the sample counts minus the sample blank counts.

Results

- Using the linear graph paper provided, plot a curve of ^{125}I counts for the five standards against the β -TG concentration printed on the standard vials. Draw a smooth curve through the means of the duplicate points to give a standard curve. Grossly aberrant counts should be rejected. A typical curve is given in Figure 1.
- Using the mean of duplicate counts for the unknowns, read off their β -TG concentrations from the standard curve.

Figure 1. β -TG RIA Kit Typical Standard Curve



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The following sample collection protocol has been developed to allow preparation of platelet poor plasma samples as simply and conveniently as possible. The protocol is a modification of that recommended by Ludam and Cash. The system employs a mixture of anticoagulant and antiplatelet reagents which, in combination with reduced temperature, minimizes β -TG release by platelets during processing (*in vitro*) and separates platelets and other formed elements of blood from the plasma by centrifugation techniques. In this way, the measured β -TG value reflects the *in vivo* circulating level of β -TG at the time of sampling. The tubes containing the antiplatelet and anticoagulant agents are provided in a separate container which has been designed to act as an ice bath for cooling samples.

Sample Collection and Preparation

1. Tap the sampling tube gently on a hard surface to ensure that the liquid contents are at the bottom.
2. Place a mixture of CRUSHED ICE AND WATER in the base of the tube box and replace the rack containing the sampling tubes. Sufficient water is required to ensure good thermal contact and rapid cooling of the blood samples.
3. Using standard venipuncture techniques, withdraw a 2.5 ml blood sample using a 20g x 1" needle and syringe (preferably polystyrene). EVACUATED SAMPLE COLLECTION TUBES MUST NOT BE USED FOR COLLECTION OF BLOOD SAMPLES. A good venipuncture with minimal venous occlusion must be performed. When blood for other tests is drawn at the same time, no more than 10ml of blood should be collected. The total time taken for the venipuncture should be less than two minutes.
4. IMMEDIATELY after collection, the needle should be removed from the syringe and 2.5ml of blood should be transferred gently to a pre-cooled labeled sampling tube supplied with the kit. The tube is marked at 2.5ml for convenience.
5. Stopper the tube and mix the contents by gentle inversion two or three times and IMMEDIATELY cool the sample by placing it in the rack in the cooling bath.

THE BLOOD SAMPLE MUST BE MIXED WITH THE ANTICOAGULANT AND ANTIPLATELET REAGENT MIXTURE AND COOLED AS RAPIDLY AS POSSIBLE AFTER COLLECTION.

6. Allow sufficient time for the sample to cool. Ensure that the ice remains in the cooling container throughout this time.
7. Centrifuge the sample at 1500-2000g and 2-4°C for 30 minutes. Centrifugation must be carried out within three hours after collection.
8. After centrifugation, carefully remove the top 0.5 ml of plasma using a pipette with a disposable plastic tip. Transfer this sample to a separate labeled specimen tube.

The sample for assay can be stored at room temperature for up to 24 hours or at 2-4°C for up to seven days. Storage for longer periods should be at -20°C for up to 4 weeks. If the sample is stored frozen, it should not be repeatedly frozen and thawed. Thawed samples should be mixed well prior to use.

Interfering Substances

Samples should be checked for radioactivity prior to assay since significant radioactivity may cause interference with the assay depending on the behavior of the radioactive material in the assay system. Significant activity may be pres-

ent if the count rate for 50 μ l patient sample is greater than 10% of the counts in the highest standard (after assay)

Activity may be significant if the patient has received 125 I fibrinogen for thrombosis detection. Preferably, samples for β -TG measurement should be obtained prior to or 24 hours or more after 125 I fibrinogen administration.

Procedure

Materials Provided

Standard β -TG in Buffer — 5 vials
 β -TG 125 I — 1 vial
 Anti- β -TG Serum — 1 vial
 Ammonium Sulfate Solution — 1 vial
 Blood Sampling Tubes — 24
 Tube Rack/Cooling Bath Container
 Data Sheet

Materials Required

For Sample Collection and Separation:

Tube marker (waterproof)
 Refrigerated centrifuge capable of 2000g — horizontal head
 Crushed ice

5 or 10ml syringes — preferably polystyrene
 Needles — 20 gauge (1" length preferred)

Sample storage tubes

For Assay:

Precision pipettes — 50, 200 and 500 μ l sizes
 Assay tubes — 12 x 75mm polystyrene tubes, round bottomed (other types of tubes are not recommended)

Vortex mixer

Centrifuge capable of 1000g

Absorbent towels

Decantation racks

Gamma scintillation counter

Graduated 10ml glass pipette

Tube racks

Distilled water

Details of Procedure

1. Number the assay tubes as shown in the protocol (Table 1).
2. Pipette duplicate 50 μ l aliquots of the reconstituted standards and unknowns into the assay tubes as shown in the protocol (See Note 1)
3. Pipette 200 μ l aliquots of the β -TG 125 I solution into each assay tube
4. Pipette 200 μ l aliquots of the anti- β -TG serum solution into each assay tube and vortex mix the tubes

Reagents

Each β -TG RIA Kit contains sufficient reagents for the construction of a 5 point standard curve and the assay of 19 unknowns in duplicate on one occasion, or two standard curves and a total of 14 unknowns in duplicate on two occasions or any combination of standards and unknowns to a total of 48 tubes. A standard curve must be constructed on both occasions.

If a large number of samples are to be assayed on a single occasion, reagents from two or three kits of the same lot may be pooled before use. Only a single standard curve is required in this case.

Description

1. ^{125}I β -TG (human, freeze-dried) — contains up to $2\mu\text{Ci}$, 74kBq ^{125}I in 10ml solution after reconstitution.
2. Anti- β -TG (rabbit, freeze-dried) — contains antiserum sufficient to bind at least 40% of 0.5ng β -TG in 10ml of solution after reconstitution.
3. Ammonium Sulfate Solution — contains $>25\text{ml}$ of a 3.3M solution of ammonium sulfate (sulphate).
4. β -TG (human) in Buffer (freeze-dried) — after reconstitution, each vial contains 0.5ml of a solution with β -TG concentrations of approximately 10, 20, 50, 100 and 225ng/ml. The exact values are stated on the vial labels.
5. Blood Sampling Tubes — contains anticoagulant and antiplatelet agents.

Precautions

FOR RESEARCH USE ONLY. NOT FOR USE IN DIAGNOSTIC PROCEDURES.

Caution: Radioactive Material

Not for Internal or External Use in Humans or Animals.

This radioactive material may be received, acquired, possessed and used only by physicians, clinical laboratories, or hospitals and only for in vitro clinical or laboratory tests not involving internal or external administration of the material, or the radiation therefrom, to human beings or animals. Its receipt, acquisition, possession, use and transfer are subject to the regulations and a general license of the U.S. Nuclear Regulatory Commission, or of a state with which the Commission has entered into an agreement for the exercise of regulatory authority, or license by the Canadian Atomic Energy Control Board. This kit contains radioactive material which should be handled with appropriate precautions in use and disposal.

Instructions Relating to the Handling, Use and Storage of Radioactive Materials

Introduction Into Foods, Beverages, Cosmetics, Drugs, or Medicinals, or Into Products Manufactured for Commercial Distribution is Prohibited — Exempt Quantities Should Not Be Combined.

1. Radioactive material should be stored in specially designated areas not normally accessible to unauthorized personnel.
2. Radioactive material should be used only by responsible persons in authorized areas. Care should be exercised to prevent ingestion or contact with the skin or clothing. In the event that contact is made with radioactive material, the contaminated areas should be thoroughly washed with detergent.

*The amount of radioactivity in the bequest (Bq) equal to one disintegration per second. $1\mu\text{Ci} = 37$

3. Pipetting of radioactive solutions *must not* be done by mouth.
4. No smoking, drinking, or eating should be allowed in areas where radioactive materials are used.
5. Hands should be washed after using radioactive materials.
6. Work should be carried out on a surface covered with absorbent materials.
7. Any spills of radioactive material should be cleaned immediately and all contaminated materials disposed as radioactive waste. Contaminated surfaces should be washed with a detergent.

Reconstitution

Standards

Tap the vials of freeze-dried buffer standards to dislodge any large particles from the stoppers. Remove the tear-off aluminum closures, carefully remove the rubber stoppers and place them inverted on a clean surface. Gently add 500 $\pm 10\mu\text{l}$ of freshly distilled water onto the inside surface of each of the vials and replace the stoppers. Allow to dissolve at room temperature for two minutes. Invert each vial gently to remove any particles of desiccant from the cap and to obtain a homogeneous solution. Avoid vigorous agitation and foaming.

Antiserum and ^{125}I

Reconstitute the freeze-dried antiserum and ^{125}I β -TG reagents with $10 \pm 0.2\text{ml}$ distilled water. Ensure that homogeneous solutions are obtained by gentle inversion. Avoid vigorous agitation and foaming.

Storage

The β -TG RIA Kit should be stored at $2-4^\circ\text{C}$.

The reagents may be stored at $2-4^\circ\text{C}$ for up to one week after reconstitution.

The ammonium sulfate solution can be stored at $2-4^\circ\text{C}$ or at room temperature.

Before use on a second occasion, reagents should be allowed to come to room temperature and be mixed to ensure homogeneity.

Availability

From stock.

Expiration

The expiration date is stated on the package and will normally be 3-6 weeks from the date of shipment.

Do not use the kit after the expiration date shown on the label.

Preparation and Storage of Samples

Sample preparation is the critical stage in this assay. THE FOLLOWING PROTOCOL MUST BE STRICTLY ADHERED TO. Improper sample preparation may result in elevated β -TG levels. The user should be familiar with this protocol prior to collecting any samples for assay.

Serum samples are not acceptable for this test because β -TG is released by platelets during the blood clotting process, resulting in serum β -TG levels in the range $(0.25\mu\text{g/ml})$. In addition, samples of normal plasma are unsuitable because platelet release can occur during the collection and processing of plasma samples, resulting in β -TG levels of $(1.10\mu\text{g/ml})$. Platelet poor plasma processed so as to minimize platelet release during handling must be used for this assay.

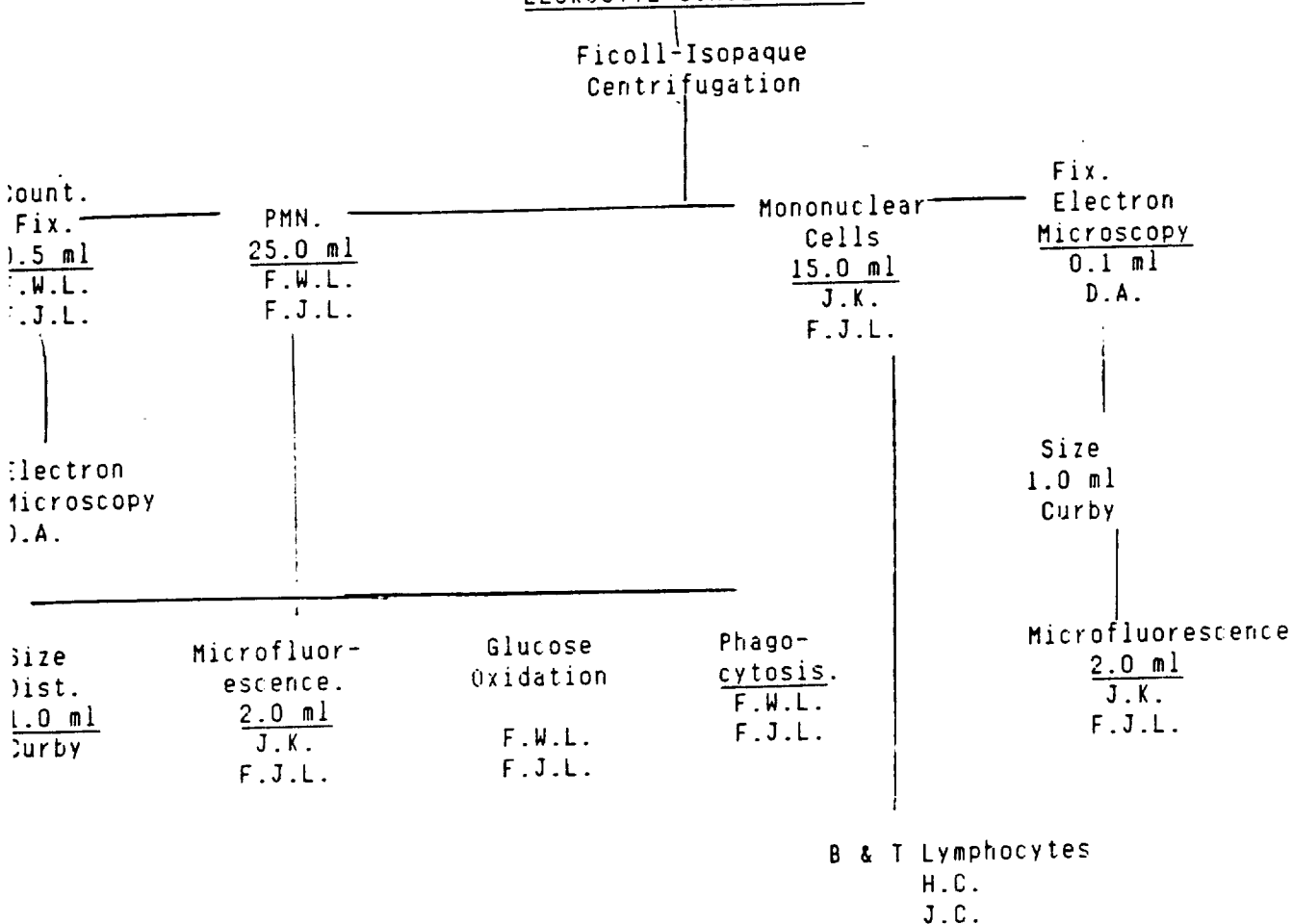
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4.3 LEUKOCYTES

OUTLINE OF ISOLATION AND TESTING

13 Bags of Sedimented Buffy Coat (150.0 ml each, 9 in space, 9 on ground)
6 each in PVC-DEHP, PVC-TOTM, and Polyolefine Plastic

LEUKOCYTE CONCENTRATES



LEUKOCYTES

Abstract

Buffy coat white cells will be isolated from whole blood and sedimented with Dextran. Leukocyte concentrates will be centrifuged, and the supernatants containing Dextran and platelets discarded. Nine bags of leukocytes will be reconstituted to 150 ml with buffered plasma and sent into space. An additional nine bags will be maintained on the ground for an equivalent storage period. All bags will be coded and randomized post-flight.

Polymorphonuclear cells without further purification will be examined by electron and fluorescence microscopy, and electronically for volume distribution. Phagocytic and respiratory burst activity also will be assayed.

Mononuclear cells will be separated from leukocyte concentrates with Ficoll-Hypaque. They will be examined without further purification by electron and fluorescence microscopy, electronically for size distributions and by flow cytometry for differential counts and T cell stimulation responses.

A. POLYMORPHONUCLEAR CELLS

1. Preparation of Leukocyte Concentrates.

Using the standard procedures for obtaining platelets, 75.0 ml of buffy coats will be harvested into a 300 ml transfer pack. Via a sterile connector 15.0 ml of sterile 2% Dextran will be added from a stock previously filtered through 0.44 μ membrane filter. The bag contents are mixed briefly manually and the bag suspended upside down. Sedimentation of red cells is allowed to proceed for 45 minutes at room temperature. The red cells are carefully drained via the original sterile connector and discarded. The bag containing the leukocyte concentrate is centrifuged in a RC-3 Sorval centrifuge in a blood bank head HG at 4°C and 300 x g for 8-10 minutes. The supernatant fluid containing Dextran and platelets is expressed and discarded. The white cell pellet is resuspended to 150 ml in the bag with PBS buffered plasma (1:1).

2. Microfluorescence of Cytoplasm and Nuclei of Granulocytes.

The reaction of granulocytes with fluorescein diacetate (FDA) and ethidium bromide (EB) in Hank's balanced salt solution (HBSS) without calcium and magnesium is performed. About 1×10^6 granulocytes are mixed in proportions of 0.05 to 0.25 ml with 0.50 ml of a mixture of fluorescein diacetate and ethidium bromide. Wet mounts are made at room temperature and the cells are viewed within one minute with an Olympus Vanox transmission microscope. A green exciter filter (G533) is used for identification of the granulocytes and fluorescence is viewed after switching to a UV exciter filter (Schott BG-12) and a blue barrier filter (Schott OG-530). Esterase activity in the cytoplasm of granulocytes is measured as the percent of cells showing green fluorescence of the fluorescein liberated from fluorescein diacetate. The percentage of cells with red fluorescence nuclei due to uptake of ethidium bromide is also recorded. Two hundred cells are counted.

The percentage of viable cells is obtained from the fraction of green cells to the total of green plus red fluorescent cells.

3. Determination of Size Distributions

Cell suspensions containing 100,000 to 150,000 granulocytes per ml are sized in a Coulter Model ZH with a C-1000 Channelyzer and an X-Y recorder. Prior to testing the granulocyte samples, the Coulter aperture is flushed with the buffered solution that is used to dilute the granulocytes. The sample volume is 0.1 ml. All solutions are rendered particle-free by filtration through both the 0.45 and the 0.20 micron filters in a Coulter filtration system or with 0.22 micron Falcon filters. A 7084 aperture is used. This is set at an amplification of 1, an aperture current of 4, an exclusion of 4, a lower threshold of 5, and an upper threshold of 99. The settings for the Channelyzer are: count control on External, count range 1 K, base channel threshold 1, and window width 100. Calibration is with 2.0 micron and 9.8 micron polystyrene particles. Granulocyte counts are made by integration of the number of cells within the granulocyte distribution. Granulocyte counts in whole blood and other samples are also made with a Coulter Counter Model F blood cell counter after lysis of red blood cells with Zap-Isoton. Red blood cell contamination of isolated granulocytes is counted as the difference in counts between the nontreated and Zap-Isoton treated aliquots at a 500 fold dilution.

4. Phagocytosis Assay.

a. Preparation of ^{125}I labeled *Staph aureus*.

Staphylococcus aureus (IgG-sorb) is reconstituted with 10 ml cold distilled H_2O yielding a 10% (v/v) cell suspension in phosphate buffered saline (PBS) (150 mM NaCl, 40 mM phosphate pH 7.2 containing 0.05% azide). A 0.5 ml aliquot of the stock suspension is washed three times with 3 ml of PBS pH 7.0 at 2200 rpm for 10

minutes at 4°C and resuspended to 0.3 ml. Three hundred ul of washed IgG-sorb is then added to a 10 x 100 glass tube which contains 50 ug of Iodogen evaporated on the vessel bottom and the original tube is washed with 100 ul buffered saline (pH 7.0) and combined. Five hundred uCi of ^{125}I in 100 ul of buffered saline is then added, gently rotated to initiate contact with the Iodogen pellet and allowed to incubate with occasional rotation on ice bucket under constant rocking. One half milliliter of 150 mM KI is added to stop the reaction. The labeled cell suspension is washed four times with 3 ml volumes of buffered saline (pH 7.0) and the cpm/ul of *Staph aureus* and the ^{125}I incorporated is determined.

b. Phagocytosis of Iodinated *Staphylococcus aureus* by Human Neutrophils.

In each sample to be tested 100 ul of a 20-fold diluted ^{125}I -*Staph aureus* suspension is added to 12 x 75 polypropylene tubes containing (in duplicate) 0.5 ml of fresh normal autologous, heterologous, or heat-inactivated serum. Controls will contain buffered saline plus Mg^{++} and Ca^{++} . The suspensions are incubated at 37°C for 30 minutes with constant shaking to keep cells suspended. The opsonized particles are then washed 2X in ice cold buffered saline at 2200 rpm for 10 min at 4°C. After the final centrifugation, the cell pellets are resuspended to 0.4 ml in KRP pH 7.4 plus 5 mM glucose. Neutrophils (5×10^6) are added to each tube and the final volume adjusted to 0.5 ml. The suspensions are mixed continuously for 20 minutes at 37°C. Ice cold buffered saline is added to stop ingestion and the cells are washed to remove free uningested particles (150 x g for 10 minutes at 4°C). The cells are then counted in a gamma counter. The phagocytes indices are determined as counts of radioactivity ingested per 10^6 cells (cpm in normal sera - cpm in PBS = cpm ingested by *Staph aureus*).

5. Glucose Oxidation by Human Leukocytes. $C^{14}O_2$ from ^{14}C -1 labeled glucose.

Human leukocytes from control and 5 day old test samples in a concentration of $1-1.5 \times 10^7$ cells/ml will be suspended in 3 mls of Kreb's Ringer Phosphate pH 7.4 containing 1 mM glucose and 0.5 uCi of $[1-^{14}C]$ glucose (approx. 50 mCi/mM). Then 1-2 ug of PMA or f-MLP (10^6 M), both soluble stimuli for neutrophils will be added. The suspension will be incubated in a 25 ml side-arm erlenmeyer flask stoppered by a cap fitted with a cup containing 0.2 ml of hyamine hydroxide. After a 30 min incubation at $37^\circ C$ in a shaking water bath, the mixture will be acidified by injection of 1 ml of 5 N H_2SO_4 . The cell suspension will be equilibrated for an additional 30 min and then the cup removed and the contents assayed for radioactivity by liquid scintillation counting using a Beta Counter.

The amount of $^{14}CO_2$ released from $[1-^{14}C]$ glucose will be expressed as the percent of (control) unstimulated leukocytes. Fresh preparations of purified human neutrophils and leukocyte rich plasma will also be included as controls for each test group.

6. Electron Microscopy - Diana Ausprunk.

Granulocytes and mononuclear cells will be studied by transmission and scanning electron microscopy following fixation in 2.5% glutaraldehyde buffered to pH 7.4 with sodium cacodylate. The samples will be transported to Boston at $4^\circ C$ following post fixation in 1% osmium tetroxide in sodium cacodylate buffer.

- a. Scanning: An aliquot of fixed leukocytes will be passed through a nucleopore filter. The filters with attached cells will be dehydrated in ethanol. Following deposition of gold-palladium the cells will be examined and photographed in a JSM-35 scanning microscope. This will provide a three-dimensional view of the shape and surface structure of the cells.

b. Transmission: An aliquot of fixed cells will be dehydrated in increasing concentration of ethanol. The dehydrated cells are infiltrated with plastic resin, cut on an ultra microtome, placed on grids and stained. The stained sections are studied and photographed in a JEM 100 B electron microscope. This will provide a high resolution view of the structure of the cell cytoplasm.

c. Whole Mount Preparation: A suspension of cells is spread on a Formvar-coated electron microscope grid and processed by critical point drying. The entire complement of organelles and cytoskeleton within each cell can be observed simultaneously. Any defects in cell spreading or cytoplasmic organization can be readily detected.

7. Cell shape analysis of granulocytes and mononuclear cells - W.M. Curby.

a. Electronic Analysis: The shape of stored white cells will be analyzed using the Curby Biodetector (CBD). The ability of each cell type to maintain size and shape against known osmotic gradients over fixed time intervals will be monitored.

b. Photomicrographs of wet slide preparations will be made of cells from each formed element population before and after the application of gradients.

Stop action photomicrographs of each cell population will be done using Xenon strobe, point source light illumination. The cells will be photographed in hanging drop slide preparations to obviate distortion associated with the adherence of a blood cell to a glass microscope slide.

B. MONONUCLEAR CELLS*

1. Isolation of Mononuclear Cells

Ten ml of leukocyte concentrate are overlaid onto 3 ml of Lymphoprep in a 15 ml conical tube and centrifuged at 1500 rpm (400 x g) for 30 min at 20°C. The top 5 ml is removed and discarded. The mononuclear cells are removed from the interface between Lymphoprep and saline and placed in a 15 ml test tube. The remaining fluid above the pellet is discarded. All cells are kept on ice (2°C).

2. Tests on Mononuclear Cells

Monocytes and lymphocytes in Lymphoprep isolates will be tested for size and membrane integrity by microfluorescence as described in A (Polymorphonuclear Cells). Lymphocyte differential counts will be made with an EPICS cell sorter using monoclonal antibodies to specific cell types. Helper-suppressor T cell ratios will be determined as well as the T cell stimulation response.

*Note: Protocols for mononuclear cell studies from collaborators, Carter, Taylor and Yunis are in discussion.

*Protocols for mononuclear cell testing are being developed collaboratively with Drs. Carter and Taylor.

WHITE CELL EXPERIMENT

MATERIALS AND EQUIPMENT

Processing Buffy Coat to isolate PMS and MNES
Quantity per unit processed

MATERIALS:

2 Sampling site coupler (Fenwal 4C2405)
50 ml syringes (BD 5663)
1 box 18 g needles 1.5" x 18 g (100/box) (BD 5196)
2 Styrofoam rack for 15 ml and 50 ml T.T.
DEXTRAN Hi M.W. Sigma D-52S1 200,000-300,000 M.W.
NaCl
10 ml pipettes 25/unit
2 pipette bulbs
15 and 50 ml polypropylene test tubes/conival tubes
Lymphoprep 1.077 gr/ml 1 case (100 ml bottles)
PBS pH 7.4 + glucose 3 liters
glass microscope slides 75mm x 25 1 box
glass microscope slips 22mm x 22m 1.5 thickness 1 box
Fluorescein Diacetate powder
Ethidium Bromide powder
Pooled Plasma 3.0 liters
12 x 75 mm test tubes polypropylene with caps 2 cases
Coulter accuvettes for cell counting & ZAP-oglobin II (Azide free) 2 boxes
Coulter chart paper for C-1000 xy plotter
Glass Pasteur pipette 5.75" disposable glass
Plasma expressor and 3 hemostats & 1 pair scissors
Isoton, Coulter, 1 cube

EQUIPMENT

Sorvall RC 3B refrigerated centrifuge with head (6 place)
IEC CRU 5000 centrifuge with head #253
Refrigerator for blood storage 4-6°C range & reagents
Fluorescence microscope w/Schott OG-530 barrier and Schott BG-12 filters
Water bath, shaking, 37°C
Coulter ZH counter, C1000 channelyzer and X-Y plotter
Vortex mixer
Magnetic stirrer and magnetic stir bars
Balance - triplebeam
Vacuum and vacuum trap set up with small air pump
Double beam spectrophotometer, Cuvettes
J isotope counter (gamma)

SPACE, PLUMBING AND ELECTRICAL REQUIREMENTS FOR
WHITE CELLS' STORAGE EXPERIMENT

Fabian Lionetti
F. Wm. Luscinskas
James Cohn

For Microscopy:

Water bath, 37°C, 20" x 15" x 6" approximately
Olympic Vanox fluorescence microscope
Illuminator

Bench space 2' x 6' x 2.5' = 14 sq'
2 Electrical outlets 110 V
1 sink, stainless, 20" x 10" x 10"

For Cell Counting and Volume Studies:

Coulter Model ZH
Coulter Channelyzer
Coulter X-Y Recorder
Dade Diluter
Vortex Mixer
Coulter Filtration System

Bench Space 2' x 10' x 3' = 20 sq'
6 Electrical outlets 110V
Coulter S+4 Diff. Counter 10 sq'

LEUKOCYTES
SUMMARY OF BENCH SPACE, PLUMBING AND ELECTRICAL REQUIREMENTS

Bench Space: 70 sq'

40 sq' Work

30 sq' For Equipment

Desks: (2) 2' x 5' = 20 sq'

Sinks: (2) 2' x 2' x 1.5'

Outlets: (16) 110 V

Special power requirements for Coulter S+4 Diff. Counter:

An independent, protected circuit three wire outlet, 105-125 Vac.
single phase input power, 20 A. 1500 W power consumption.

14.4 Electron Microscopy Protocol

Part I. To be completed at on-site facility

A. Fixation of RBC

- 1) 10 ml whole blood
- 2) ghosts

B. Fixation of Platelets

- 1) 5 ml platelet concentrate
- 2) ADP-stimulated platelets
- 3) serotonin-stimulated platelets

C. Fixation of Leukocytes

D. Preparation of Platelet Whole Mounts

E. Preparation of Leukocyte Whole Mounts

Part II. To be completed at Children's Hospital, Boston

A. Transmission Electron Microscopy of RBC, Platelets and Leukocytes

- 1) postfixation, dehydration, embedding
- 2) sectioning and microscopy

B. Scanning Electron Microscopy of RBC, Platelets and Leukocytes

C. Freeze-fracture of RBC

D. TEM & SEM of Whole Mounts of Platelets and Leukocytes

EQUIPMENT AND SUPPLIES FOR ELECTRON MICROSCOPY

Equipment:

Tabletop centrifuge
Top loading balance
Refrigerator
CO₂ incubator
Phase contract microscope
Tank of 5% CO₂: 95% air mixture

Supplies:

Pipettes (1, 5 & 10 ml)
Pasteur pipettes (6", plastic)
Conical test tubes (plastic w/caps) - 100 ml + 50 ml
Parafilm (roll)
Scissors
Timer (1 hr)
Racks for test tubes
Gold grids (formvar coated)
Petri dishes (35 mm, sterile, tissue culture plastic)
Forceps
Hemocytometer
Pocket calculator
Graduated cylinders (50 + 100 ml)
Reagent bottles

Reagents:

Distilled H₂O

Gluteraldehyde (10%, nitrogen sealed vials)

Sodium cacodylate

CaCl₂

Sucrose

Dulbecco's PBS

Eagle's medium

30 mOsm buffer

DETAILED EM PROTOCOL

Part I. To be completed at on-site facility

A. Fixation of RBC (9 samples of 5 ml each)

100 ml of 1% gluteraldehyde in Eagle's medium (pH 7.0) will be added to each 5 ml sample of RBC. After 30 min at room temperature, RBCs are centrifuged at room temperature and 100 rpm for 10 min. After removing the supernatant, the cells are washed in Eagle's medium, recentrifuged and resuspended in 100 ml of Eagle's medium containing 6% sucrose. The cells are stored at 4°C and transported to CHMC. Red cell ghosts (obtained from Dr. Jacobson) will be similarly prepared except that 30 m Osm buffer replaces Eagle's medium.

B. Fixation of Platelets (9 samples of 2 ml each)

40 ml of 2.5% gluteraldehyde in 0.1 M sodium cacodylate buffer (pH 7.4) will be added to each 2 ml sample of platelets. After 30 min at room temperature, platelets are centrifuged at room temperature and 5000 g for 7 min. After removing the supernatant, the platelets are washed in 0.1 M sodium cacodylate buffer, recentrifuged and resuspended in 40 ml of cacodylate buffer containing 6% sucrose. The cells are stored at 4°C and transported to CHMC. Platelets stimulated by ADP and serotonin will be similarly prepared.

C. Fixation of Leukocytes (9 samples 3 ml each)

60 ml of 2.5% gluteraldehyde in 0.1 M sodium cacodylate buffer (pH 7.4) will be added to each 3 ml of leukocytes. After 30 min at room temperature, cells are centrifuged at room temperature, 100 rpm for 10 min. After removing the supernatant, the cells are washed in cacodylate buffer, recentrifuged and resuspended in 60 ml of cacodylate buffer containing 6% sucrose. The cells are stored at 4°C and transported to CHMC.

D. Preparation of Platelet Whole Mounts (9 samples of 1 ml each)

Gold electron microscopy grids will be prepared at CHMC in advance of arriving at Cape Kennedy by cleaning them in dilute nitric acid, coating the grids with a thin film of 0.7% Formvar plastic and evaporating a thin layer of carbon onto the Formvar film. Platelets are washed free of plasma proteins and resuspended at a concentration of 50,000/ml in 0.9% NaCl. One drop of platelet suspension from each sample is placed onto each of four grids laying on a piece of Parafilm contained in a 35 mm Petri culture dish. The dishes are covered and placed in a humidified CO₂ incubator (5% CO₂: 95% air) at 37°C for 10-20 min. Each grid is removed from the incubator, rinsed in Dulbecco's phosphate buffered saline and placed in a vial containing 1 ml of 2.5% gluteraldehyde in 0.1 M sodium cacodylate buffer (pH 7.4). After fixation for 30 min at room temperature, the grids are rinsed in cacodylate buffer and then stored at 4°C in 1 ml of cacodylate buffer containing 6% sucrose.

E. Preparation of Leukocyte Whole Mounts (9 samples of 1 ml each)

Gold electron microscopy grids are prepared as described for platelet whole mounts. Leukocytes are diluted to a concentration of 200,000 cells/ml in 0.9% saline. A drop of cell suspension is placed onto each of four grids contained in a Petri dish as described for platelets. The covered dishes are placed in the 37°C incubator for 2 hr and then fixed and processed using identical methods described for the platelet whole mounts.

Part II. To be completed at Children's Hospital, Boston

A. Transmission Electron Microscopy of RBC, Platelets and Leukocytes
(27 samples)

One ml of each sample of fixed RBCs, RBC ghosts, platelets and leukocytes will be centrifuged into a pellet and postfixes in buffered 1% osmium tetroxide (in Eagle's medium for RBC, 30 mOsm buffer for ghosts, 0.1 M sodium cacodylate buffer for platelets and leukocytes) for 60 min at 4°C. After washing 3X in distilled water, cells are dehydrated in increasing concentrations of ethanol (70%, 80%, 95%, 100%) for 5 min in each alcohol. The dehydrated cells are placed in 2 changes of propylene oxide (20 min each) and embedded in plastic resin (Polybed). Sections 1 mm thick are stained in methylene blue-azure II examined by light microscopy and photographed using Kodak Plus-X film. Thin sections are cut with diamond knives on a Reichert ultramicrotome, doubly stained with uranyl acetate and lead citrate and examined and photographed in a JEM 100 B electron microscope.

B. Scanning Electron Microscopy of RBC, Ghosts, Platelets and Leukocytes (27 samples)

0.25 ml of each sample of fixed RBCs, ghosts, platelet and leukocytes will be passed through a Nucleopore filter (0.45 μ m pore size) by pressure filtration. Filters with attached cells are dehydrated in ethanol and critical point dried from liquid CO₂. A thin layer gold-palladium is deposited on the cells in a sputter coater. After attaching the filters to specimen stubs, the cells will be examined and photographed in a JEOL JSM-35 scanning electron microscope.

C. Transmission and Scanning Electron Microscopy of Whole Mounts of Platelets and Leukocytes

Whole mounts of fixed platelets and leukocytes are postfixes for 5 min at 4°C 0.5% osmium tetroxide buffered to pH 7.4 with 0.1 M sodium cacodylate. The cells are dehydrated in ethanol and critical point dried from liquid CO₂. A thin layer of carbon is evaporated over the cells. The pre-

parations will be studied, by transmission electron microscopy and photographed in a JEM 100 B electron microscope. The microscope is equipped with a goniometer stage so that pairs of stereophotographs will be obtained by tilting the stage $\pm 6^\circ$ - 7° from the horizontal. After transmission electron microscopy, the same specimen will be coated with gold-palladium in a sputter coater and examined in the scanning electron microscope.

D. Freeze Fracture of RBCs and Ghosts

One ml aliquots of fixed RBCs and ghosts are centrifuged into a pellet, washed with Dulbecco's PBS and the pellet resuspended in 20% glycerol in PBS for 15 min. Glycerinated cells will be fractured in a Bolzers apparatus at -115°C and the fracture face will be replicated by evaporating platinum and carbon onto its surface. Replicas are cleaned by floating over methanol and chlorine bleach to digest away the cells. The replicas are transferred to copper grids and examined in the transmission electron microscope.

Time Frame for Completion of Electron Microscopy

Part I. Work to be completed on-site

| | |
|---|---------|
| 1. Fixation of all cells | 2 hours |
| 2. Centrifugation and washing of cells | 3 hours |
| 3. Resuspension of cells in storage buffer | 2 hours |
| 4. Packing of specimens for transport to Boston | 3 hours |

TOTAL 10 hours

Part II. Work to be completed at Children's Hospital, Boston

The Initial Blood Storage Experiment

NASA-IBSE

Protocols to be Performed at the Launching Site

Immunology

Introduction

The following protocol describes the tests to be done by UMMC staff at the Kennedy Space Center in the context of the NASA-IBSE (Initial Blood Storage Experiment). The protocol is divided into two sections, I Materials and II Methods. Section I will describe the essential laboratory space required including the major equipment. Some of the equipment already exists at KSC laboratories and is available for our use. Other equipment is provided by the manufacturer for the period of the experiment. The remainder is to be either brought in by UMMC staff or ordered to be sent to KSC directly. These items are indicated by *. In the Materials section there will be a list of the laboratory supplies and the reagents and buffers required for processing the RBC and platelet specimens for the immunological assays. Special reagents needed for individual tests will be given in the Methods section, under the heading of each special test. The II section will describe the following procedures to be conducted at KSC:

- 1.0 Glycerolization and Freezing of RBC
- 2.0 Processing Platelet Concentrate Samples
- 3.0 Quantitative Antiglobulin Consumption (QAC) for Platelet-Associated C3c and C3d
- 4.0 QAC for Platelet-Associated IgG

I. MATERIALS

LABORATORY SPACE AND MAJOR EQUIPMENT:

A -80°C freezer must be available in the laboratory at the site.

A 4°C cold room with sufficient space and accessible electrical outlet to accommodate a 0.5 amp, 110 V powered test tube agitator* (12'x9'x24').

A sink with 2 faucets for the attachment of two aspirator pumps.

An RC-5 centrifuge (Sorval) with SS-34 rotor.

Bench space (approx. 15'x2.5') to accommodate the following:

2 IEC centr-7 centrifuges equipped with #210 rotors and carriers for 13 mm, 16 mm, and 30 mm diameter test tubes.

*2 vortex mixers.

An electronic cell counter (capable of counting platelets) and associated reagents and supplies.

Crushed ice supply.

Dry ice supply.

LABORATORY SUPPLIES:

*Adjustable Finn timers™ and tips.

*Test tube agitator.

*20 and 5 ml graduated syringes.

*4" and 250' roll of parafilm.

*250 ml beakers.

*2000 ml and 500 ml graduated cylinders.

*Plastic and glass transfer pipets.

*Insulated containers for sample transport.

*Electronic 3-channel timer.

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*Test Tubes.

- *12 x 75 mm glass tubes.
- *16 x 100 mm siliconized glass tubes (vacutainer red tops).
- *30 x 100 mm plastic centrifuge tubes.
- *2 ml and 13 ml capped plastic freezing tubes.

*Assorted reagent containers.

*Tape, markers, etc. for labeling.

*Racks for test tubes.

*500 ml volumetric flasks.

REAGENTS, BUFFERS:

100 1 reagent H₂O

*Dry chemicals, pre-weighed, for making the following:

0.9 g/dl NaCl with 10 mM phosphate, 0.1 g/dl NaN₃, pH 7.2 (PBS).

PBS containing 0.5 g/dl bovine serum albumin (PBSA).

PBSA containing 0.15 g/dl Na₂EDTA (PBSA-EDTA).

Glycerolyte™ 57 solution.

II. METHODS

1.0 PROCESSING WHOLE BLOOD SAMPLES FOR SHIPMENT TO UMMC

Reference: I.O. Szymanski, J.M. Harper, P.R. Odgren, C.R. Valeria. Freezing red blood cells prepared for quality control of antiglobulin sera. *Transfusion* 21:498-501, 1981.

1.1 Sample required: 30 ml whole blood

1.2 Procedure

1.2.1 The sample(s) is divided into two 50 ml centrifuge tubes and washed 3 times with PBS and the RBC packed after final wash.

1.2.2 After the final wash add to the packed cells a volume of glycerolyte equal to 0.4 times the volume of the packed cells. The

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glycerolyte is added slowly and with constant gentle mixing on vortex mixer.

1.2.3 The RBC and glycerolyte are allowed to equilibrate at room temperature for 10 min.

1.2.4 Slowly and with constant gentle mixing on vortex mixer, a volume of glycerolyte equal to 1.6 times the original volume of the packed RBC is added.

1.2.5 The contents of both tubes are then pooled, mixed and aliquoted as follows:

Two aliquots of at least 1.5 ml each (for detection of IgG and complement by direct agglutination tests)

Four aliquots of at least 6 ml each (for quantitation of C3 and IgG on RBC membrane)

1.2.6 10 min. after the final addition of glycerolyte, the samples are frozen at -80°C .

1.2.7 Samples are stored in -80°C freezer until they are transported to UMMC, at which time they are packed in an insulated container with dry ice, and shipped.

Man hours for the processing of whole blood sample: one person can handle 18 samples in approximately 6 hours.

2.0 PROCESSING PLATELET CONCENTRATE SAMPLES

2.1 Sample required: A 10 ml sample is obtained on the day of launch, and 18 5 ml samples of platelet concentrate are obtained on the day of return of the satellite. A larger sample is required initially since platelet-bound complement is low in that sample.

2.2 Platelets are pelleted by centrifugation at 2000 RCF for 7 minutes, the supernatant plasma decanted, and they are then washed 6 times with 5 ml PBSA-EDTA.

2.3 After the final wash, platelets are suspended with a volume of PBSA-EDTA sufficient to yield a concentration of approximately 1×10^6 /microliter in day 0 sample and approximately 5×10^5 /microliter in day 5 samples. The exact platelet count in the washed platelet suspension is determined using the cell counter.

3.0 QUANTITATIVE ANTIGLOBULIN CONSUMPTION TEST (QAC) FOR PLATELET-ASSOCIATED C3c AND C3d

Reference: I.O. Szymanski, R.E. Swanton, and P.R. Odgren. Quantitation of the third component of complement on stored red cells. *Transfusion* 24:194-197, 1984.

3.1 Additional reagents required:

3.1.1 Anti-C3 (C3c-specific): Goat anti-human C3, IgG fraction, obtained from Atlantic Antibodies (Scarborough, ME). For this assay, it is diluted 1:100,000 in PBSA, and has been given lot #C3(G).

3.1.2 Anti-C3d: Goat anti-human C3d, obtained from Netherlands Red Cross (Amsterdam). Heteroagglutinins have been removed from this serum by absorption with bromelin-treated RBC. For this assay, it is diluted 1:5,000 in PBSA and has been given lot #17.

3.1.3 Zymosan Standards (ZyC3) consist of zymosan A particles which have been coated with C3, washed, and diluted in PBSA. This ZyC3 has been analyzed for C3 quantity as described before and sets of aliquots have been prepared for both C3c and C3d assays. Each set consists of ten 1.0 ml aliquots, each containing a dif-

ferent concentration of Zyc3. For C3d standardization, the C3 content in aliquots range from 1.81×10^{10} molecules/ml through 7.24×10^{11} mol/ml. For C3c standardization, the C3 range is from 8.06×10^9 mol/ml through 1.6×10^{11} mol/ml.

3.2 Procedure

3.2.1 Sample dilution:

3.2.1.1 Day 0 suspension is tested both undiluted and diluted 1/2 in PBSA-EDTA.

3.2.1.2 Day 5 suspensions are tested undiluted and diluted 1/2 and 1/5 in PBSA-EDTA.

3.2.2 Sample pipetting: 1 ml of each platelet suspension and required dilution(s) of Zyc3 is dispensed into a 2 ml tube in triplicate. One set is pipetted for C3c, another one for C3d.

3.2.3 0% neutralization controls: 1 ml PBSA is dispensed into 20 2 ml tubes.

3.2.4 Antibody pipetting: To each tube of the C3 set (i.e., including dilutions of various platelets, three sets of Zyc3 standards, and 10 PBSA controls) exactly 1 ml of anti-C3c will be added; to each tube of the C3d set exactly 1 ml of anti-C3d will be added.

3.2.5 The tubes are all tightly capped, placed on test tube agitator, and allowed to incubate overnight (18-24 hrs.) at 4°C.

3.2.6 The incubated samples are centrifuged for 30 minutes at 12,000 RCF and the supernates transferred to appropriately labeled 2 ml freezing tubes.

3.2.7 The supernates are frozen at -80°C and stored until shipped to UMMC in insulated containers with dry ice.

4.0 QACT FOR PLATELET-ASSOCIATED IgG

Reference: N. Levitan, R. Tano, I. Szymanski. An autoanalyzer test for the quantitation of platelet-associated IgG. Submitted for publication.

4.1 Additional reagents required:

*4.1.1 Anti-IgG (Fc specific), obtained from Atlantic Antibodies (Scarborough, ME) was absorbed with bromelin-treated RBC to remove heteroagglutinins and given BBR Lot #2. For this assay it is diluted 1:1,000,000 with PBSA.

*4.1.2 IgG Standards consist of Beckman ICS Calibrator I serum (Beckman Instruments) which has been calibrated against a World Health Organization IgG Standard. It is diluted with PBSA (in two-fold fashion) to a minimum of 8 dilutions, each 1.0 ml in volume. The concentration of IgG in these 8 standards is to range from 31.8 ng/ml to 0.24 ng/ml. Four "0%" neutralization targets are prepared also by dispensing 1.0 ml of PBSA into each of four tubes.

*4.1.3 IgG Control consists of Beckman ICS Calibrator serum which is diluted in PBSA (in two-fold fashion) to 4 dilutions, each 1.0 ml in volume. The concentration of IgG in these controls is to range from 14.2 to 1.77 ng/ml.

4.1.4 Platelet Dilution: Platelet suspension (from 5.3 above) is diluted to contain approximately 200,000 platelets/ml in PBSA-EDTA.

4.2 Procedure

4.2.1 Anti-IgG neutralization by the IgG Standards: To each of the 1.0 ml standards (from 7.1.2 above), add 1.0 ml of the 1:1,000,000 dilution of anti-IgG (from 7.1.1 above).

4.2.2 Anti-IgG neutralization by Platelets: Set up the consumption of anti-IgG by testing four concentrations of the platelet suspension (7.1.4 from above) as follows:

Dispense 1.0 ml of PBSA into each of four 12 x 75 mm tubes. To one of each of the tubes add 10, 20, 30 and 40 ul of the platelet suspension. Next add 1.0 ml of the diluted anti-IgG reagent (4.1.1 from above) and mix thoroughly. (Since these are set up in duplicate, the final number of tubes per test is eight.) (It has been found that with such small volumes of the platelet suspensions it is not necessary to take into account the platelet volume when making these dilutions.)

4.2.3 Anti-IgG neutralization by the IgG controls: To each of the 1.0 ml controls (from 4.1.3 above), add 1.0 ml of the diluted anti-IgG reagent (4.1.1 from above).

4.2.4 After the above have been set up, all tubes are stoppered and incubated at 4°C for at least 18 hr with constant, gentle agitation on the tube shaker (2.1). It is important that the unknowns be incubated simultaneously with the standards. After incubation, the tubes are centrifuged at 2000 x g for 10 min and the supernates transferred to the 2.0 ml plastic freezing tubes, capped tightly, and placed in the -80°C freezer. These are eventually transported in the frozen state to UMMC where the assay will be completed.

TEST TO BE PERFORMED FOR NASA AT
THE UNIVERSITY OF MASSACHUSETTS MEDICAL CENTER

INTRODUCTION:

The purpose of these studies will be to measure red blood cell (RBC) and platelet associated immunoglobulin and complement on samples of human blood which have been stored in a micro-gravity environment and on the Earth's surface. This is done with the intent of establishing if deposition of these plasma proteins increases (or decreases) upon the storage of human blood in space. A sensitive, automated direct agglutination assay will be used to determine relative amounts of immunoglobulin (Ig)G and the complement fragments, C3a, C3c and C3d on intact RBC. Precise quantitation of IgG, C3c and C3d on intact RBC and platelets will be made with the quantitative antiglobulin consumption test (QACT). In addition, the QACT for IgG will be performed on isolated RBC stroma and lysates. Both of these assays use the Autoanalyzer (Technicon Corp., Tarrytown, NY) and are described in detail below.

1.0 The automated direct agglutination assay: This assay detects the presence of specific protein(s) on the surface of RBC and is based on the phenomenon of agglutination of said RBC by specific antibody. The results, expressed in percent agglutination, are proportional to the amount of protein of the RBC. Therefore, it is possible to express relative concentrations of cell-bound protein among different RBC populations. This assay will be performed as described in detail elsewhere (1). Briefly, washed RBC, suspended to a 10% hematocrit in Ficoll and 0.9% NaCl are introduced continuously into an Autoanalyzer manifold together with 1.0% polyvinylpyrrolidone K-90 (PVP) in 0.9% NaCl while appropriately diluted antibodies (to each of the four proteins) are sampled discontinuously. RBC bearing the specific protein agglutinate in the presence of corresponding antibody. The agglutinated RBC are

removed by gravity and the remaining unagglutinated RBC hemolyzed with Triton X-100 and water. The OD₅₅₀ of the lysate is recorded and the percent agglutination which reflects directly the amount of protein on the RBC membrane is then calculated.

1.1 Materials.

1.1.1 Phosphate buffered saline (PBS): 0.9% NaCl, 0.1% NaN₃, buffered to pH 7.2 w/0.01 M sodium phosphate.

1.1.2 Manifold cleaning solution: 2.5% urea in 0.05% NaOH in H₂O.

1.1.3 0.5% Ficoll (Pharmacia) in PBS.

1.1.4 0.5% bovine serum albumin (BSA) in PBS.

1.1.5 1.0% polyvinylpyrrolidone K-90 (PVP) in PBS containing 2 drops of Tween-20 (Technicon) per 50 ml of solution.

1.1.6 Antisera to:

IgG (Fc specific) (Atlantic Antibodies, Scarborough, ME) (diluted 1:5000)

C3a (Cappel) (diluted 1:200)

C3c (Atlantic Antibodies) (diluted 1:2000)

C3d (Neatherlands Red Cross) (diluted 1:200)

1.1.7 Lysing solution: 1.0% Triton X-100 (Sigma Chemical Co., St. Louis, MO) in H₂O with 0.1% NaN₃.

1.1.8 The glassware, instruments and facilities for performing the assay are available at the UMMC Blood Bank Research Laboratories.

1.2 Samples: Samples of glycerolyzed RBC will be thawed and deglycerolyzed (Appendix). The recovered RBC will be washed in PBS and suspended to a 10.0% hematocrit in 0.5% Ficoll solution.

1.3 Procedure:

1.3.1 An Autoanalyzer (Technicon, Tarrytown, NY) is outfitted with a sampler, tubing, manifold, reservoirs, pump, and colorimeter as shown in Figure 1.

1.3.2 The reservoirs for PBS and Triton are filled.

1.3.3 The platen on the proportionating pump is engaged and the sampling probes placed in the cleaning solution.

1.3.4 The pump, colorimeter and recorder are turned on.

1.3.5 After 20-30 min the probes are placed in PBS containing 2 drops of Tween-20 per liter of solution for 20-30 additional min.

1.3.6 The electronic scale is adjusted to read between 00 and 100% T(T).

1.3.7 The antibody probe is placed in the BSA solution. The Analyzer is allowed to run for 10-20 min. The appropriate sampling probes are placed in the RBC suspension (which is agitated continuously with a magnetic stirrer and always maintained in an ice bath) and PVP solution. The antibody probe is engaged to the sampler and sampling is begun. The reaction products will reach the photo cell of the colorimeter in approximately 25 min.

1.3.8 After the fluids have reached the colorimeter, the baseline is adjusted with the "baseline" knob to 80-90%T.

1.3.9 After the baseline is established, sampling of the antibody solutions follows.

1.3.10 The change in optical density from the baseline for each sample is determined from the chart paper and the % agglutination calculated from the following formula:

$$\frac{\%T \text{ of baseline} - \%T \text{ of the sample}}{\%T \text{ of the baseline}} \times 100 = \% \text{ agglutination}$$

The percent agglutination is proportional to the number of molecules of antigen on the surface of the RBC and provides a measure of the relative quantities of membrane bound protein among different cell populations.

2.0 The quantitative antiglobulin consumption test (QACT): This assay quantitates the molecules of a specific protein (IgG, C3c or C3d) that accumulate on or within RBC and platelets. In this test varying amounts of test sample or known standards are incubated with a known amount of specific antibody. Thereafter, the antibody remaining in the supernate is measured by direct agglutination of indicator RBC coated with the specific protein. The percent neutralization is calculated and dose response curves constructed. The total number of molecules of protein per cell is then determined by using the dose response curves, Avogadro's number and the known molecular weight of the protein.

2.1 Materials:

2.1.1 All of the material described in section 1.1.

2.1.2 RBC products to be tested:

2.1.2.1 Intact RBC: RBC samples received will be deglycerolyzed (Appendix), washed three times with PBS, resuspended to about 50% hematocrit and the total cell count determined electronically with a Coulter Counter (Coulter Electronics, Hialeah, FL).

2.1.2.2 Lysates for internal IgG determinations: For each 1.0 ml of the 50% RBC suspension, 0.5 ml of a 0.5% solution of digitonin in PBS is added while gently mixing on a Vortex mixer. 1.5 ml of PBS is added and after 2 min the mixture is centrifuged at 2000 x g for 7 min and about 1.0 ml of the supernatant hemolysate is transferred to dialysis tubing (12,000 to 14,000 molecular weight

sieve) and dialysed against 250 volumes of PBS for 1 hr. (The pellet is saved for the preparation of the stroma as outlined below). The hemoglobin concentration of the lysate is determined on a Coulter Counter and kept at 4°C until tested for IgG.

2.1.2.3 RBC stroma: The pellet from step 2.1.2.1 is washed with PBS five times or until there is no visible hemoglobin and resuspended in an equal volume of PBS. A 100 ul aliquot is mixed with 2.0 ml of 1.0% sodium dodecylsulfate in H₂O for 10 min. The OD of the solution at 260 and 280 nm is obtained and the total stromal protein determined from the following formula:

$$(21(1.55(OD_{280}) - 0.77(OD_{260})) = \text{mg protein/ml stromal suspension}$$

$$\langle (8.1 \text{ mg/ml} = 10^{10} \text{ RBCm stroma}) \rangle$$

2.1.3 Indicator, IgG coated RBC: Approximately 250 ml of packed group O Rho positive RBC are combined with 60 ml of anti-Rho (Ortho Diagnostics, Raritan, NJ). After 30 min at 37°C the RBC are glycerolyzed (2), aliquoted and stored at -80°C.

2.1.4 Indicator, C3-coated RBC: These indicator RBC are prepared as previously described (3). These are also glycerolyzed and stored at -80°C.

2.1.5 IgG soluble standard: Beckman ICS Calibrator serum (Beckman Instruments, Irvine, CA) with known concentration of IgG is diluted with PBS to a concentration of approximately 30 ng/ml. This stock solution is aliquoted and stored at -80°C. Working standards (to range in concentration from 30 to 0.025 ng/ml are prepared by serially diluting the stock solution.

2.2 Procedure for the QACT:

2.2.1 The first step of this assay consists of combining different amounts of the antigens (intact RBC, lysates, soluble IgG standards and ZyC3) with a known amount of antiserum according to the following table:

| Antigen | Volumes of the antisera | | |
|----------------|-------------------------|----------------------------|-----------------------------|
| | Anti-IgG 4 ml (1:2M) | Anti-C3c 0.5 ml (1:50K) | Anti-C3d 0.5 ml (1:4.5K) |
| 50% RBC | 20* 40 | - - | - - |
| 15% RBC | - | 588 | 588 |
| 7.5% RBC | - | 541 | 541 |
| Stroma | 200 100 | - - | - - |
| Lysate | 20 40 | - - | - - |
| IgG standards | 20 | - | - |
| ZyC3 standards | - | 5000 | 500 |

*Volume of antigen in μ l.

These mixtures are incubated overnight at 4°C with gentle agitation whereafter they are centrifuged and the supernatants tested for remaining antibody activity against the appropriate indicator RBC by the direct agglutination test described in 1.0.

NOTE ON PLATELET QACT: The incubation of the platelets with the antibodies will have been done on NASA site and the frozen supernatants containing the remaining antibody will be tested at UMMC.

2.2.2 Calculation of results: The percent agglutination is calculated and a dose-response curve depicting the percent neutralization of antibody by known standard is constructed. Molecules per cell are determined by using the dose response curves, Avogadro's number and the molecular weight of the given antigen (IgG, C3c, or C3d).

Appendix:

Deglycerolization of frozen RBC:

1. Frozen RBC are thawed at 37°C.
2. 12% NaCl is added* in a volume equal to 25% of the glycerolyzed sample and allowed to equilibrate at room temperature for 5-10 min.
3. 1.6% NaCl is added in a volume equal to the glycerolyzed sample and allowed to equilibrate for at least 2 min.
4. The sample is centrifuged and the supernatant decanted.
5. 1.6% NaCl is added as in step 3.
6. Step 4 is repeated.
7. PBS is added in a volume equal to the original glycerolyzed sample volume and allowed to equilibrate for at least 2 min.
8. Step 4 is repeated.
9. Step 7 is repeated.
10. Step 4 is repeated.
11. The RBC are resuspended in the desired diluent.

*All solutions are added slowly and with constant stirring with Vortex stirrer.

References cited:

1. Szymanski, I.O., Huff, S.R., Delsignore, R.: An autoanalyzer test to determine immunoglobulin class and IgG subclass of blood group antibodies. *Transfusion* 22:90 (1982).
2. Technical Manual of the American Association of Blood Banks. J.B. Lippincott, Philadelphia, pp. 60-61 (1981).
3. Chaplin, H. Jr., Freedman, J., Massey, A., Monroe, M.C.: Characterization of red blood cells strongly coated in vitro by C3 via the alternative pathway. *Transfusion* 20:254 (1980).
4. Szymanski, I.O., Odgren, P.R.: Measurements of fragments of the third component of human complement on erythrocytes by a new immunochemical method. *Vox Sang.* 46:9 (1984).

Figure 1.

INITIAL BLOOD STORAGE EXPERIMENT

Biophysics Protocol

All data taking to be completed at on-site facility.

A. Total Cellular Count

1. From each red blood cell sample, a 10^{-2} ml aliquot will be added to a 10.0 ml of 0.9% w/v particle free saline and mixed by capping and inversion ten times. From this stock solution, 1.0 ml will be taken and added to 9 ml of the 0.9% particle free saline solution. This final concentration will be read directly on the Curby Bio-detector (CBD) using a short tunnel 5×10^{-2} mm diameter aperture. Cell counts and cell shape analysis will be made on 5×10^{-2} ml volumes of the final concentration fluid.
2. White blood cell counts will be made on a final solution concentration containing 10^{-2} ml of each white blood cell sample in 10.0 ml of 0.9% particle free saline. Cell counts and cell shape analysis will be made on 5×10^{-2} ml volumes of the final concentration fluid.
3. Platelet counts concentration will be arrived at by first making a stock solution containing 10^{-2} ml of the platelet preparation in 10 ml of 0.9% particle free saline. 2 ml of the stock solution is then added to 8 ml of the 0.9% saline to make the final counting solution. All pipetting will be done using automatic microliter adjustable pipets and certified pipet tips.
4. For all cell counts, the CBD will be set to count one peak per generated pulse. The size will be determined by placing the add one count in the memory storage channel equal to the maximum pulse height within the pulse. All pulse height measurements will be made when a negative pressure equivalent of 7.0 inches of mercury exists across the aperture tunnel of the 5×10^{-2} mm aperture.

B. Cell Shape Analysis

1. Pulse shape analysis will be done using 1 inch of mercury pressure differential across the aperture. Aliquots of each sample will be drawn through the aperture, and in sequence, reading first the peak height and then the pulse height generated by each cell at 4, 8, 16 and 32 microseconds after the initial rise of the voltage pulse. All samples will be normalized by allowing the cell frequency distribution to be accumulated in the computer memory until the highest peak in the storage channel vs. frequency distribution reaches 100 counts full scale.
2. Distributions will be plotted by the CBD plotter and filed for analysis.

C. Red Blood Cell Action to Applied Unit Stress

1. Pulse height data will be collected as in Section A1. Immediately after the initial reading 1 ml of particle free, pyrogen free distilled water will be added to the sample and peak readings will be repeated once every 2 minutes until a peak shift of more than 10 channels is observed between subsequent readings.
2. The cell counts and size frequency data will be recorded and printed out by the CBD.

D. Photomicrographs

1. Photomicrographs of each sample will be taken. Tungsten source light and Xenon source light will be used.
2. Two photomicrographs of each sample of red blood cells will be made using a Nikon Model S-Ke Microscope, an anachromatic dark contrast optical combination, viz., a 100X DLL achromatic objective and a matching 100 annular diaphragm in the focal plane of the condenser. Dark-low-low phase contrast images will be recorded on 35 mm, 1000 ASA speed print film. The field coverage film frame indexing will be done using a 10^{-2} mm etched spacing stage micrometer. Red blood cells will also be photographed using a 100X plan apochromat lens and direct lighting under similar tungsten light source conditions.
3. A slide for each sample will be prepared by dropping exactly 10^{-2} ml of sample onto a cleaned microscope slide and tipping a 1 cm x 1 cm cover slip onto the sample so that the center of the cover slip lands on the center of the drop. A drop of immersion oil having a refractive index of 1.515 will be centered on top of the cover slip. No oil will be used to fill in below the microscope slide and the microscope condenser.
4. Samples of platelets and white blood cells will be applied to make up microscope slide preparations in the manner described in D3. DLL phase contract pictures will be made from these samples.
5. Red blood cell samples will also be prepared for hanging drop stop-action photomicrographs by placing a 10^{-2} ml of a one in ten diluted drop of each sample on a 1 cm x 1 cm cover slip and carefully inverting the cover slip and placing it over the center of a depression slide. The cover slip will be waxed in place.
6. Stop action (100X objective) photomicrographs will be made using E. Leitz Microscope Equipment and a Leica "Microibso" unit with a 1/3 lens to reform the image on the camera film plane. The light source will be a Xenon Strobe Unit which is centered along the optical axis of the microscope 15 centimeters below the objective focal plane of the microscope. Five centimeters above the strobe light bulb a diaphragm having a 4 mm opening is placed centered on

the optical axis parallel to the film plane. (This diaphragm effectively forms the point for formation of a new wave front.) No condenser is needed. A columnated tungsten light source and 45° removable mirror is placed above the diaphragm when a focusing light is needed. The microscope is focused onto the center of the hanging drop of sample, the mirror is removed and a flash picture is made of the cells drifting in the drop.

7. Color print film (35 mm) will be ASA matched to the optical system. As before a 10^{-2} mm spacing stage micrometer will be used for frame identification and size indexing on the films.

15.0 ACQUISITION OF DATA

16.0 ANALYSIS OF DATA

17.0 SAFETY PROCEDURES

17.1 Dr. Fabian Lionetti from The Center for Blood Research, is the Safety Officer.

INITIAL BLOOD STORAGE EXPERIMENT

Schedules for Blood Collection, Component Preparations and Distribution

1.0 The purpose of this document is to define the timelines and responsibility for IBSE personnel to collect, prepare, pool and distribute whole blood and its components in preparation for loading into flight hardware and shuttle launch. The activity described in this document is detailed in Annex 8 of the Payload Integration Plan.

2.0 Manpower Requirements

| | |
|---------------|------|
| M.S. Jacobson | CHMC |
| D. Van Pelt | CBR |
| S. Kevy | CHMC |
| F. Lionetti | CBR |
| T. Curran | CBR |
| C. Smith | CFBB |
| A. Scanlon | CHMC |

3.0 Schedule

The activity of blood collection, component preparation and sample distribution is scheduled in relation to the shuttle launch. The schedule is developed on the basis of a launch on December 18, 1985 at 7:00 a.m. The activity begins at twenty-five hours prior to launch (T-25, 6:00 a.m., 12/17/85). The schedule of the activity is as follows:

See following page for table

4.0 Activity Record

The activity related to blood collection, platelet and leukocyte preparation will be entered on the attached record sheets.

| <u>Code</u> | <u>Time</u> | <u>Activity</u> | <u>Personnel Responsible</u> |
|-------------|---|---|------------------------------|
| 3.1 | T-25 (6:00 a.m. 12/17) | Begin blood collection | May Jacobson |
| 3.2 | T-24.5 (6:30 a.m. 12/17) | Begin platelet and leukocyte preparation | " |
| 3.3 | T-22.5 (8:30 a.m. 12/17) | Begin whole blood pooling | " |
| 3.4 | T-22 (9:00 a.m. 12/17) | Completion of blood collection | " |
| 3.5 | T-20 (11:00 a.m. 12/17) | Completion of whole blood pool | " |
| 3.6 | T-20 (11:00 a.m. 12/17) | Completion of individual leukocyte units | " |
| 3.7 | T-20 (11:00 a.m. 12/17) | Begin distribution of whole blood | " |
| 3.8 | T-20 (11:00 a.m. 12/17) | Begin leukocyte pooling & distribution | " |
| 3.9 | T-19.5 (11:30 a.m. 12/17) | Completion of individual platelet units | " |
| 3.10 | T-19.5 (11:30 a.m. 12/17) | Begin platelet pooling & distribution | " |
| 3.11 | T-19 (12:00 p.m. 12/17) | Completion of distribution of whole blood, leukocytes, platelets into designated bags | " |
| 3.12 | T-19 (12:00 p.m.. 12/17) | Begin packing blood bags into 8 boxes for transportation to KSC | " |
| 3.13 | T-18.5 (12:30 p.m. 12/17) | Completion of packing blood bags | " |
| 3.14 | T-18 (1:00 p.m. 12/17) | Departure of blood from CFBB | " |
| 3.15 | T-17 (2:00 p.m. 12/17) | Arrival of blood at KSC | " |
| 3.16 | T-17 (2:00 p.m. 12/17) | Begin loading of dewars | " |
| 3.17 | Continuation of pre-launch activity as described in "Prelaunch Closeout Procedures" (ADL Reference C-53282-45). | | |

ACTIVITY RECORD - WHOLE BLOOD COLLECTION

| <u>UNITS COLLECTION</u> | <u>TIME STARTED</u> | <u>TIME COMPLETED</u> | <u>OPERATOR</u> |
|-------------------------|---------------------|-----------------------|-----------------|
| 1ST DONOR | | | |
| 2ND DONOR | | | |
| 3RD DONOR | | | |
| 4TH DONOR | | | |
| 5TH DONOR | | | |
| 6TH DONOR | | | |
| 7TH DONOR | | | |
| 8TH DONOR | | | |
| 9TH DONOR | | | |
| 10TH DONOR | | | |
| 11TH DONOR | | | |
| 12TH DONOR | | | |
| 13TH DONOR | | | |
| 14TH DONOR | | | |
| 15TH DONOR | | | |
| 16TH DONOR | | | |
| 17TH DONOR | | | |
| 18TH DONOR | | | |
| 19TH DONOR | | | |
| 20TH DONOR | | | |
| 21ST DONOR | | | |
| 22ND DONOR | | | |
| 23RD DONOR | | | |
| 24TH DONOR | | | |
| 25TH DONOR | | | |

ACTIVITY RECORD - WHOLE BLOOD COLLECTION

| <u>UNITS COLLECTION</u> | <u>TIME STARTED</u> | <u>TIME COMPLETED</u> | <u>OPERATOR</u> |
|-------------------------|---------------------|-----------------------|-----------------|
| 26TH DONOR | | | |
| 27TH DONOR | | | |
| 28TH DONOR | | | |
| 29TH DONOR | | | |
| 30TH DONOR | | | |
| 31ST DONOR | | | |
| 32ND DONOR | | | |
| 33RD DONOR | | | |
| 34TH DONOR | | | |
| 35TH DONOR | | | |
| 36TH DONOR | | | |
| 37TH DONOR | | | |
| 38TH DONOR | | | |
| 39TH DONOR | | | |
| 40TH DONOR | | | |

WHOLE BLOOD POOLING AND DISTRIBUTION

| ACTIVITY | TIME STARTED | TIME COMPLETED | OPERATOR |
|---|-----------------|-------------------|----------|
| POOLING OF FIRST 6 UNITS OF WHOLE BLOOD (FIRST POOL) | | | |

MIXING 1ST

MIXING 2ND

MIXING 3RD

MIXING 4TH

MIXING 5TH

MIXING 6TH

POOLING OF SECOND 6 UNITS OF WHOLE
BLOOD (SECOND POOL)

MIXING 1ST

MIXING 2ND

MIXING 3RD

MIXING 4TH

MIXING 5TH

MIXING 6TH

POOLING OF FIRST AND SECOND WHOLE
BLOOD POOL

MIXING 1ST

MIXING 2ND

MIXING 3RD

MIXING 4TH

MIXING 5TH

MIXING 6TH

DISTRIBUTION INTO DESIGNATED BAGS

PLATELET PREPARATION

| ACTIVITY | TIME STARTED | TIME COMPLETED | STATUS OF PREPARATION | RESPONBILE PERSONNEL |
|------------------------------------|-----------------|-------------------|--------------------------|-------------------------|
| PREPARATION OF INDIVIDUAL UNITS | | | | |

| | | | | |
|------|------------------|--|--|--|
| UNIT | PRP Preparation | | | |
| | Packed platelets | | | |
| | Resting | | | |
| | Rotation | | | |

| | | | | |
|------|------------------|--|--|--|
| UNIT | PRP Preparation | | | |
| | Packed platelets | | | |
| | Resting | | | |
| | Rotation | | | |

| | | | | |
|------|------------------|--|--|--|
| UNIT | PRP Preparation | | | |
| | Packed platelets | | | |
| | Resting | | | |
| | Rotation | | | |

| | | | | |
|------|------------------|--|--|--|
| UNIT | PRP Preparation | | | |
| | Packed platelets | | | |
| | Resting | | | |
| | Rotation | | | |

| | | | | |
|------|------------------|--|--|--|
| UNIT | PRP Preparation | | | |
| | Packed platelets | | | |
| | Resting | | | |
| | Rotation | | | |

| | | | | |
|------|------------------|--|--|--|
| UNIT | PRP Preparation | | | |
| | Packed platelets | | | |
| | Resting | | | |
| | Rotation | | | |

LEUKOCYTE PREPARATION

| ACTIVITY | TIME STARTED | TIME COMPLETED | STATUS OF PREPARATION | RESPONBILE PERSONNEL |
|------------------------------------|-----------------|-------------------|--------------------------|-------------------------|
| PREPARATION OF INDIVIDUAL UNITS | | | | |
| UNIT 1 | | | | |
| UNIT 2 | | | | |
| UNIT 3 | | | | |
| UNIT 4 | | | | |
| UNIT 5 | | | | |
| UNIT 6 | | | | |
| UNIT 7 | | | | |
| UNIT 8 | | | | |
| UNIT 9 | | | | |
| UNIT 10 | | | | |
| UNIT 11 | | | | |
| UNIT 12 | | | | |
| UNIT 13 | | | | |
| UNIT 14 | | | | |
| UNIT 15 | | | | |
| UNIT 16 | | | | |
| UNIT 17 | | | | |
| UNIT 18 | | | | |
| UNIT 19 | | | | |
| UNIT 20 | | | | |
| UNIT 21 | | | | |
| UNIT 22 | | | | |
| UNIT 23 | | | | |
| UNIT 24 | | | | |

INITIAL BLOOD STORAGE EXPERIMENT
PRE-LAUNCH CLOSEOUT PROCEDURES
(Revision 1.0)

submitted to
The Center for Blood Research
Boston, Massachusetts

submitted by
Arthur D. Little, Inc.
Cambridge, Massachusetts

December 31, 1985
ADL Reference C-53282-45

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1.0 PURPOSE

The purpose of this document is to define the procedures for Arthur D. Little, Inc. (ADL), personnel to "close-out" the flight and ground-based lockers containing the Initial Blood Storage Experiment (IBSE) in preparation for the shuttle launch.

2.0 SCOPE

The scope of this document is to describe the hardware-related activities and the loading procedures as they interface with the hardware. The scientific aspects of the loading are described in the IBSE Scientific Protocol and in Annex 8 of the Payload Integration Plan. Performance checkout of the IBSE hardware is described in the Operating, Maintenance and Handling Procedures.

3.0 MANPOWER REQUIREMENTS

The following IBSE personnel will be involved in the IBSE closeout:

- D. W. Almgren, Arthur D. Little, Inc. - Engineering Manager
- E. J. Boudreau, Arthur D. Little, Inc. - Technician
- W. A. Curby, Lahey Clinic Medical Center - Science/Engineering Interface Manager
- J. Young, Jr., Lahey Clinic Medical Center - Technician
- D. Elwood, Lahey Clinic Medical Center - Technician
- M. S. Jacobson, Children's Hospital - Scientist in Charge of Blood Bag Loading
- F. Chao or D. Van Pelt, Children's Hospital - Observer

In addition to loading and closeout personnel, additional personnel will be available to:

- o observe procedures and schedule,
- o authorize proposed changes to the procedures (e.g., use of spare unit or parts, change of loading sequence, etc.),
- o record any procedure changes.
- o interface with NASA,
- o interface with Central Florida Blood Bank.

- o interface with working press,
- o interface with point of contact of closeout team (W. Curby).

The personnel for these activities include:

- E. Michel, NASA JSC
- W. Paton, NASA KSC
- D. Surgenor, Center for Blood Research
- S. Kevy, Children's Hospital
- P. Glaser, Arthur D. Little, Inc.

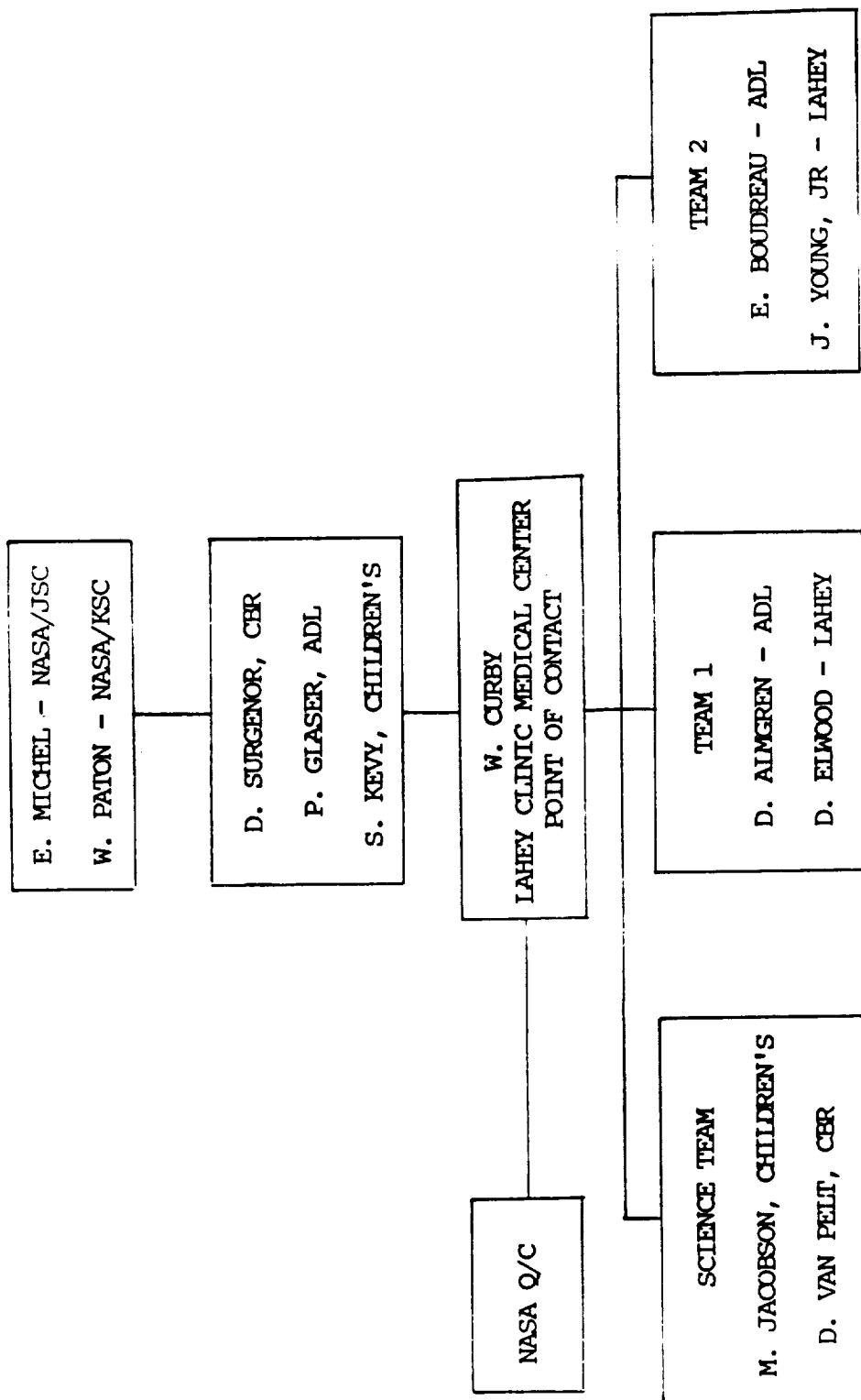
Figure 1 illustrates the IBSE organization during loading and closeout.

4.0 SCHEDULE

The activities related to IBSE loading and closeout occur in relation to the shuttle launch. This schedule assumes a launch of January 6, 1986, at 7 A.M. The schedule of events 3 days prior to launch are shown on the timeline in Figure 2 and are described below. Figure 3 shows the loading sequence of units.

4.1 Three Days Prior to Launch (1/3/86)

- 4.1.1 Post access list to closeout room.
- 4.1.2 D. Almgren and E. Boudreau check inventory of IBSE Hardware and Equipment in Hangar L.
- 4.1.3 D. Almgren and E. Boudreau check DC power supplies in Hangar L.
- 4.1.4 D. Almgren and E. Boudreau inspect each of four IBSE module, (2) P/N 4215-1200 and (2) P/N 4215-1400, in its locker.
 - 4.1.4.1 D. Almgren and E. Boudreau install Kapton air flow passages in three platelet dewars.
 - 4.1.4.2 Perform airflow check (see paragraph 6.1 of Operating, Maintenance and Handling Procedures).
 - 4.1.4.3 Set elapsed time meters and record in log book.



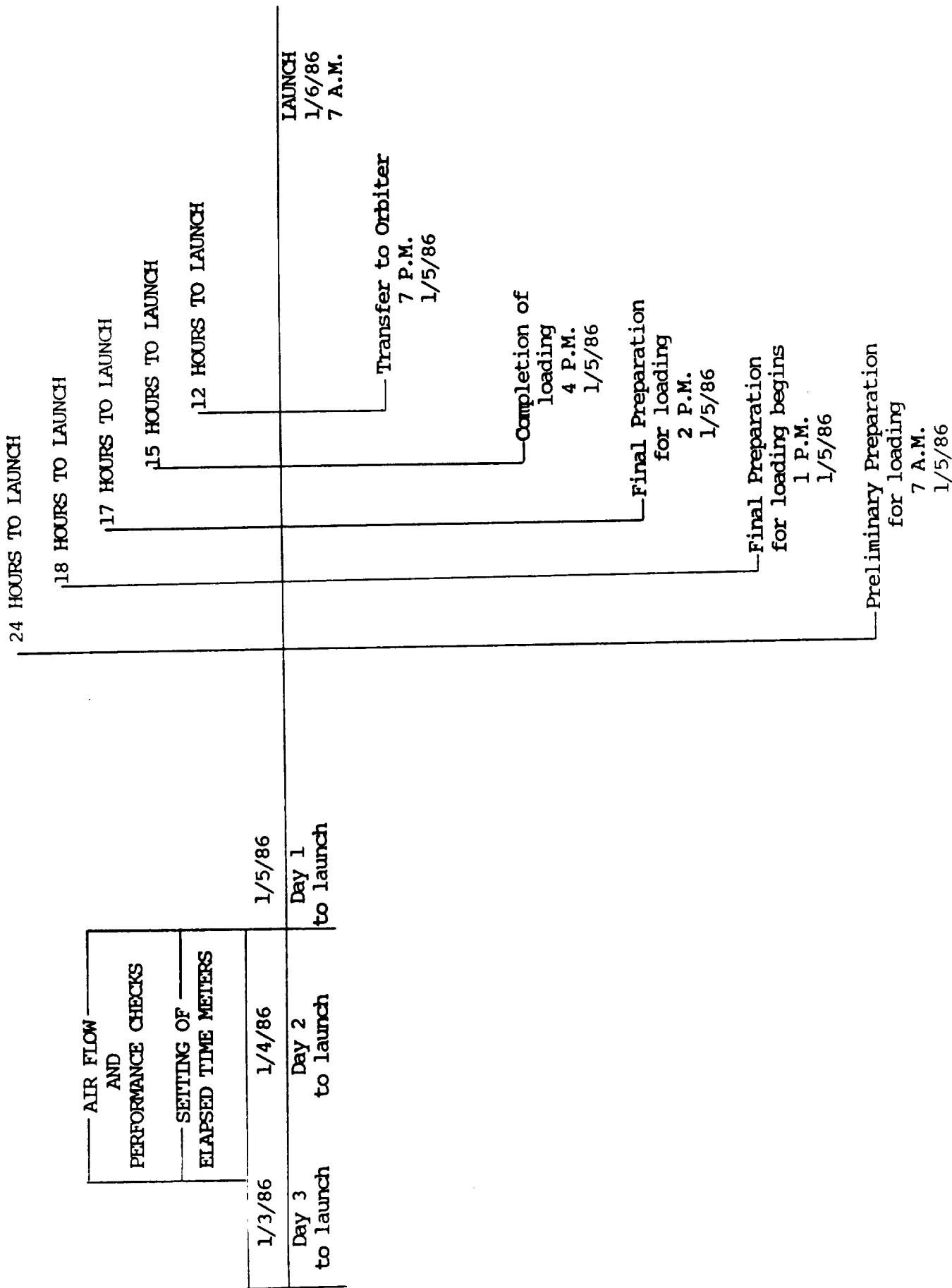
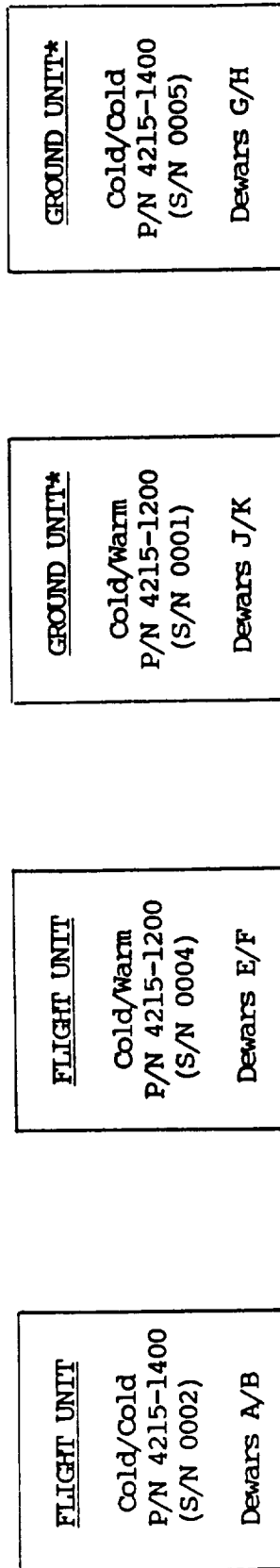


FIGURE 2 CLOCKOUT SCHEDULE



* Ground Unit Loading Sequence will be determined by need for flight substitution.

FIGURE 3

- 4.1.4.4 Closeout module without Kapton tape on perimeter of air passages.
- 4.1.4.5 Install module in locker.
- 4.1.4.6 Verify spare fuse in foam.
- 4.1.4.7 Perform thermal performance test (see paragraph 6.2 of Operating, Maintenance and Handling Procedures).
- 4.1.5 Repeat airflow and thermal tests with IBSE P/N 4215-1200 spare C/D module (S/N 0003)
- 4.1.6 D. Almgren and E. Boudreau store modules in Hanger L.
 - 4.1.6.1 Disconnect DC power supplies from IBSE modules by unplugging 25 foot cable from pigtail to DC power supply.
 - 4.1.6.2 Cover middeck lockers containing IBSE modules (located in Curby Crane) and associated 25-foot power cable with polyethylene bags.
 - 4.1.6.3 Cover spare IBSE module in cardboard box with polyethylene bag.
- 4.1.7 Power each locker for approximately 1 minute/day until launch preparations. Record data in logbooks.
- 4.2 Twenty-Four Hours Prior to Launch (1/5/86, 07:00 A.M.)
 - 4.2.1 E. Boudreau unplugs IBSE module A/B, P/N 4215-1400 (S/N 0002) from power source.
 - 4.2.2 E. Boudreau removes two electrical subpanel screws.
 - 4.2.3 E. Boudreau opens locker door to 180° position and supports it with Lucas door support.
 - 4.2.4 E. Boudreau removes top foam block.
 - 4.2.5 E. Boudreau removes belly band foam.
 - 4.2.6 E. Boudreau removes screws (approximately 16) from control box/dewar flange and verifies that the control box/bag support structure can be lifted from dewar.
 - 4.2.7 E. Boudreau repeats 4.2.1 to 4.2.6 for IBSE module P/N 4215-1200 (S/N 0004), dewars E/F.

- 4.2.8 E. Boudreau repeats 4.2.1 to 4.2.6 for IBSE module P/N 4215-1200 (S/N 0001), dewars J/K.
- 4.2.9 E. Boudreau repeats 4.3.1 to 4.3.7 for IBSE module P/N 4215-1200 (S/N 0005), dewars G/H.
- 4.3 Eighteen hours prior to launch (1/5/86 - 1:00 P.M.)
 - 4.3.1 D. Almgren and D. Elwood lift the blood storage racks out of the locker and suspend them for P/N 4215-1400 (S/N 0002), dewars A/B.
 - 4.3.2 D. Almgren removes the two thermal shields and stores them inside the dewars.
 - 4.3.3 D. Almgren covers the open dewars with polyethylene.
 - 4.3.4 Process repeated for P/N 4215-1200 (S/N 0004), dewars E/F, one thermal shield.
 - 4.3.5 Process repeated for P/N 4215-1200 (S/N 0001), dewars J/K, one thermal shield.
 - 4.3.6 Process repeated for P/N 4215-1400 (S/N 0005), dewars G/H, two thermal shields.
- 4.4 Seventeen hours prior to launch (1/5/86 - 2:00 P.M.)
 - 4.4.1 CBR personnel load blood bags into racks of P/N 4215-1400 (S/N 0002), dewars A/B, according to protocol.
 - 4.4.2 D. Almgren and D. Elwood install two thermal shields on P/N 4215-1400 (S/N 0002), dewars A/B, and apply Kapton tape to exterior of shields.
 - 4.4.3 D. Almgren and D. Elwood close out Kapton box around platelet grids.
 - 4.4.4 D. Almgren and D. Elwood lower bag support structure into dewars.
 - 4.4.5 E. Boudreau and J. Young closeout P/N 4215-1400 (S/N 0002), dewars A/B.
 - 4.4.5.1 E. Boudreau installs screws holding control box to dewars.
 - 4.4.5.2 J. Young raises the IBSE modules.
 - 4.4.5.3 E. Boudreau applies Kapton tape to perimeter of air passage.

- 4.4.5.4 J. Young lowers the IBSE modules.
- 4.4.5.5 J. Young removes eyebolt.
- 4.4.5.6 E. Boudreau puts in belly band foam.
- 4.4.5.7 E. Boudreau installs foam block while feeding subpanel through cutout in door.
- 4.4.5.8 E. Boudreau closes locker door.
- 4.4.5.9 E. Boudreau puts back subpanel screws.
- 4.4.5.10 E. Boudreau connects 25 foot cable to electric subpanel with laboratory dc power supply.
J. Young notes time in log book.
- 4.4.5.11 E. Boudreau verifies current draw and temperature.
J. Young records in log book.
- 4.4.5.12 Turnover of loaded flight module P/N 4215-1400 (S/N 0002) to NASA.
- 4.4.6 Repeat 4.4.3 to 4.4.5.12 for P/N 4215-1200 (S/N 0004), dewars E/F.
- 4.4.7 Closeout process is repeated for ground based units: P/N 4215-1200 (S/N 0001), dewars J/K, and P/N 4215-1400 (S/N 0005), dewars G/H.
- 4.5 Twelve hours prior to launch (1/5/86, 6:30 P.M.)
 - 4.5.1 NASA personnel remove P/N 4215-1400 (S/N 0002) and P/N 4215-1200 (S/N 0004) for cleaning of exterior, final weighing, transportation and installation in Orbiter.
 - 4.5.2 E. Boudreau and J. Young record temperature of units:
 - o as they set out to Orbiter.
 - o as they arrive at Orbiter,

Activity record forms for each pre-launch time step (24 hours, 18 hours, 17 hours, and 12 hours) are attached.

24 HOUR ACTIVITY RECORD

| FUNCTION | PERSON(S) | TIME | | NOTES |
|---|-----------|---------|-----------|-------|
| | | STARTED | COMPLETED | |
| 1. Unplug P/N 4215-1400 (S/N 0002) from power source (A/B) | Boudreau | | | |
| 2. Remove two electrical subpanel screws | Boudreau | | | |
| 3. Open locker to 180° pos.+ support w/ Lucas door support | Boudreau | | | |
| 4. Remove top foam block | Boudreau | | | |
| 5. Remove belly band foam | Boudreau | | | |
| 6. Remove (16) screws from control box and dewar flange | Boudreau | | | |
| 7. Verify control box/bag support structure can be lifted from dewar | Boudreau | | | |
| 8. Unplug P/N 4215-1200 (S/N 0004) from power source (E/F) | Boudreau | | | |
| 9. Remove two electrical subpanel screws | Boudreau | | | |
| 10. Open locker to 180° pos.+ support w/ Lucas door support | Boudreau | | | |
| 11. Remove top foam block | Boudreau | | | |
| 12. Remove belly band foam | Boudreau | | | |
| 13. Remove (16) screws from control box and dewar flange | Boudreau | | | |
| 14. Verify control box/bag support structure can be lifted from dewar | Boudreau | | | |

24 HOUR ACTIVITY RECORD

| FUNCTION | PERSON(S) | TIME | NOTES |
|---|-----------|-------------------|-------|
| | | STARTED/COMPLETED | |
| 15. Unplug P/N 4215-1200 (S/N 0001) from power source (J/K) | Boudreau | | |
| 16. Remove two electrical subpanel screws | Boudreau | | |
| 17. Open locker to 180° pos.+ support w/ Lucas door support | Boudreau | | |
| 18. Remove top foam block | Boudreau | | |
| 19. Remove belly band foam | Boudreau | | |
| 20. Remove (16) hot plate screws from control box and dewar flange | Boudreau | | |
| 21. Verify control box/bag support structure can be lifted from dewar | Boudreau | | |
| 22. Unplug P/N 4215-1400 (S/N 0005) from power source (G/H) | Boudreau | | |
| 23. Remove two electrical subpanel screws | Boudreau | | |
| 24. Open locker to 180° pos.+ support w/ Lucas door support | Boudreau | | |
| 25. Remove top foam block | Boudreau | | |
| 26. Remove belly band foam | Boudreau | | |
| 27. Remove (16) screws from control box and dewar flange | Boudreau | | |
| 28. Verify control box/bag support structure can be lifted from dewar | Boudreau | | |

RECORD COMPLETED BY: _____

18 HOUR ACTIVITY RECORD

| FUNCTION | PERSON(S) | TIME | | NOTES |
|--|----------------|---------|-----------|-------|
| | | STARTED | COMPLETED | |
| 1. Lift blood storage racks and suspend P/N 4215-1400 (S/N 0002) | Almgren/Elwood | | | |
| 2. Remove thermal shields (2) and store in dewar | Almgren | | | |
| 3. Cover dewars with polyethylene | Elwood | | | |
| 4. Lift blood storage rack and suspend P/N 4215-1200 (S/N 0004) (E/F) | Almgren/Elwood | | | |
| 5. Remove thermal shield (1) and store in dewar | Almgren | | | |
| 6. Cover dewars with polyethylene | Elwood | | | |
| 7. Lift blood storage rack and suspend P/N 4215-1200 (S/N 0001) (J/K) | Almgren/Elwood | | | |
| 8. Remove thermal shield (1) and store in dewar | Almgren | | | |
| 9. Cover dewars with polyethylene | Elwood | | | |
| 10. Lift blood storage rack and suspend P/N 4215-1400 (S/N 0005) (G/H) | Almgren/Elwood | | | |

18 HOUR ACTIVITY RECORD

| FUNCTION | PERSON(S) | TIME | | NOTES |
|--|-----------|---------|-----------|-------|
| | | STARTED | COMPLETED | |
| 11. Remove thermal shields (2) and store in dewars | Almgren | | | |
| 12. Cover dewars with polyethylene | Elwood | | | |

RECORD COMPLETED BY: _____

17 HOUR ACTIVITY RECORD

| FUNCTION | PERSON(S) | TIME | | NOTES |
|--|-------------------|---------|-----------|-------|
| | | STARTED | COMPLETED | |
| 1. Load blood bags into P/N 4215-1400 (S/N 0002) (A/B) | Jacobson/Van Pelt | | | |
| 2. Install two thermal shields | Almgren/Elwood | | | |
| 3. Apply Kapton tape to shields' exterior | Almgren/Elwood | | | |
| 4. Lower bag support structure into dewars | Almgren/Elwood | | | |
| 5. Install screws holding control box to dewars | Boudreau | | | |
| 6. Raise the IBSE modules | Young | | | |
| 7. Apply Kapton tape to air passage perimeter | Boudreau | | | |
| 8. Lower IBSE modules | Young | | | |
| 9. Remove eyebolts | Young | | | |
| 10. Put in bellyband foam | Boudreau | | | |
| 11. Install foam block while feeding sub-panel through cut-out in door | Boudreau | | | |
| 12. Close locker door | Boudreau | | | |
| 13. Put back subpanel screws | Boudreau | | | |

17 HOUR ACTIVITY RECORD

| FUNCTION | PERSON(S) | TIME | | NOTES |
|---|-------------------|---------|-----------|-------|
| | | STARTED | COMPLETED | |
| 15. Connect 25-foot cable to electric subpanel w/laboratory dc power supply | Boudreau | | | |
| 16. Note time of No. 15 in log book | Young | | | |
| 17. Verify current draw and temperature | Boudreau | | | |
| 18. Turnover loaded flight unit P/N 4215-1400 (S/N 0002) to NASA | Curby | | | |
| 19. Load blood bags into P/N 4215-1200 (S/N 0004) (E/F) | Jacobson/Van Pelt | | | |
| 20. Install thermal shield (one) | Almgren/Elwood | | | |
| 21. Apply Kapton tape to shield exterior | Almgren/Elwood | | | |
| 22. Close out Kapton box around platelet grid | Almgren/Elwood | | | |
| 23. Lower bag support structure into dewars | Almgren/Elwood | | | |
| 24. Install screws holding control box to dewars | Boudreau | | | |
| 25. Raise the IBSE Modules | Young | | | |
| 26. Apply Kapton tape to air passage perimeter | Boudreau | | | |
| 27. Lower IBSE modules | Young | | | |
| 28. Remove eyebolts | Young | | | |
| 29. Put in bellyband foam | Boudreau | | | |

17 HOUR ACTIVITY RECORD

| FUNCTION | PERSON(S) | TIME | | NOTES |
|---|-------------------|---------|-----------|-------|
| | | STARTED | COMPLETED | |
| 30. Install foam black while feeding sub-panel through cut-out in door | Boudreau | | | |
| 31. Close locker door | Boudreau | | | |
| 32. Put back subpanel screws | Boudreau | | | |
| 33. Connect 25-foot cable to electric subpanel w/laboratory dc power supply | Boudreau | | | |
| 34. Note time of No. 33 in log book | Young | | | |
| 35. Verify current draw and temperature | Boudreau | | | |
| 36. Turnover loaded flight unit P/N 4215-1200 (S/N 0004) to NASA | Curby | | | |
| 37. Load bag into P/N 4215-1200 (SN 0001) (J/K) | Jacobson/Van Pelt | | | |
| 38. Install thermal shield (one) | Almgren/Elwood | | | |
| 39. Apply Kapton tape to shield exterior | Almgren/Elwood | | | |
| 40. Close out Kapton box around platelet grid | Almgren/Elwood | | | |
| 41. Lower bag support structure into dewars | Almgren/Elwood | | | |
| 42. Install screws holding control box to dewars | Boudreau | | | |
| 43. Raise the IBSE Modules | Young | | | |

17 HOUR ACTIVITY RECORD

| FUNCTION | PERSON(S) | TIME | | NOTES |
|---|-------------------|---------|-----------|-------|
| | | STARTED | COMPLETED | |
| 44. Apply Kapton tape to air passage perimeter | Boudreau | | | |
| 45. Lower IBSE modules | Young | | | |
| 46. Remove eyebolts | Young | | | |
| 47. Put in bellyband foam | Boudreau | | | |
| 48. Install foam block while feeding sub-panel through cut-out in door | Boudreau | | | |
| 49. Close locker door | Boudreau | | | |
| 50. Put back subpanel screws | Boudreau | | | |
| 51. Connect 25-foot cable to electric subpanel w/laboratory dc power supply | Boudreau | | | |
| 52. Note time of No. 51 in log book | Young | | | |
| 53. Verify current draw and amps | Boudreau | | | |
| 54. Load blood bags into P/N 4215-1400 (SN 0005) (G/H) | Jacobson/Van Pelt | | | |
| 55. Install two thermal shields | Almgren/Elwood | | | |
| 56. Apply Kapton tape to shields' exterior | Almgren/Elwood | | | |
| 57. Lower bag support structure into dewars | Almgren/Elwood | | | |
| 58. Install screws holding control box to dewars | Boudreau | | | |

17 HOUR ACTIVITY RECORD

| FUNCTION | PERSON(S) | TIME STARTED/COMPLETED | NOTES |
|---|-----------|---------------------------|-------|
| 59. Raise the IBSE modules | Young | | |
| 60. Apply Kapton tape to air passage perimeter | Boudreau | | |
| 61. Lower IBSE modules | Young | | |
| 62. Remove eyebolts | Young | | |
| 63. Put in bellyband foam | Boudreau | | |
| 64. Install foam block while feeding sub-panel through cut-out in door | Boudreau | | |
| 65. Close locker door | Boudreau | | |
| 66. Put back subpanel screws | Boudreau | | |
| 67. Connect 25-foot cable to electric subpanel w/laboratory dc power supply | Boudreau | | |
| 68. Note time of No. 67 in log book | Young | | |
| 69. Verify current draw and temperature | Boudreau | | |

RECORD COMPLETED BY: _____

12 HOUR ACTIVITY RECORD

| | | TIME | |
|---|-----------|-------------------|-------|
| FUNCTION | PERSON(S) | STARTED/COMPLETED | NOTES |
| 1. Convert modules P/N 4215-1400 (S/N 0002) and P/N 4215-1200 (S/N 0004) from laboratory dc power supply to battery power source | NASA | | |
| 2. Units P/N 4215-1400 (S/N 0002) and P/N 4215-1200 (S/N 0004) are exterior cleaned, weighed, transported installed in Orbiter | NASA | | |
| 3. Temperature of P/N 4215-1400 (S/N 0002) and P/N 4215-1200 (S/N 0004) logged as they set out to, and arrive at, the Orbiter | Boudreau | | |

RECORD COMPLETED BY: _____

Initial Blood Storage Experiment

OPERATING, MAINTENANCE AND HANDLING PROCEDURES

submitted as part of the

Acceptance Data Package
Data Item 017
Contract No. NAS9-17222

submitted to

The Center for Blood Research
Boston, Massachusetts 02115

submitted by

Arthur D. Little, Inc.
Cambridge, Massachusetts 02140

October 30, 1985

ADL Reference C-53282

Table of Contents

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1.0 Purpose

The purpose of this procedure is to provide guidance and instruction to personnel who will be maintaining and operating the IBSE modules.

2.0 Scope

This procedure covers maintenance and operation of the five, flight IBSE modules.

3.0 Special Tools Required

No special tools are required.

4.0 Maintenance Schedule

Maintenance of the IBSE modules is not required on a regularly scheduled basis. It is recommended that a dewar air flow test and a thermal performance test of a module be conducted at least 60 days prior to any reflight of the module.

5.0 Condition of Environment

The thermoelectric coolers within the IBSE module reject their heat to the cooling air flowing through the module with more power dissipation required at higher air temperatures. Satisfactory operation of the modules has been measured with inlet air temperature up to 80°F. It is recommended, however, that all testing of the IBSE module be performed at ambient air temperatures between 65 and 75°F to ensure a reduced power dissipation of the module during testing.

6.0 Maintenance Procedure

Maintenance procedures for the IBSE hardware is based on two basic concerns: the dewar air flow rate and the thermal performance of the thermoelectric devices. In the following description, reference should be made to Figures 6-1 through 6-4 which are the exploded views of the IBSE hardware.

6.1 Dewar Air Flow Maintenance Procedure

The 40-50 ml/min specified air flow rate through the dewars is provided by a small, plastic, gear pump powered by a 12 v dc, brush-type motor. This pump is located in the center compartment of the control box (see assembly drawing 4215, sheet 5). The air flow rate being provided to each dewar is

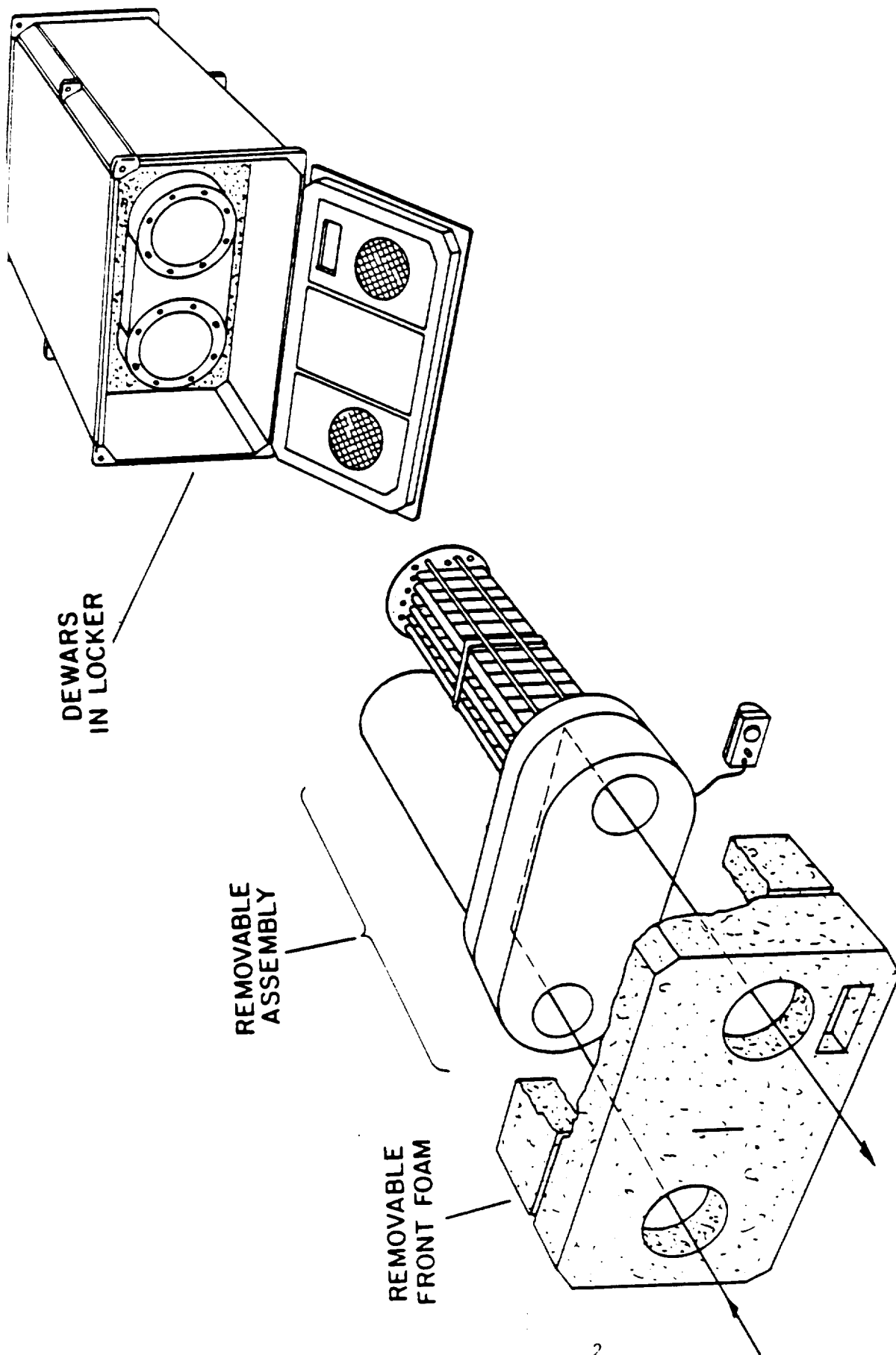


FIGURE 6-1 OVERALL VIEW OF THE COLD/WARM MODULE

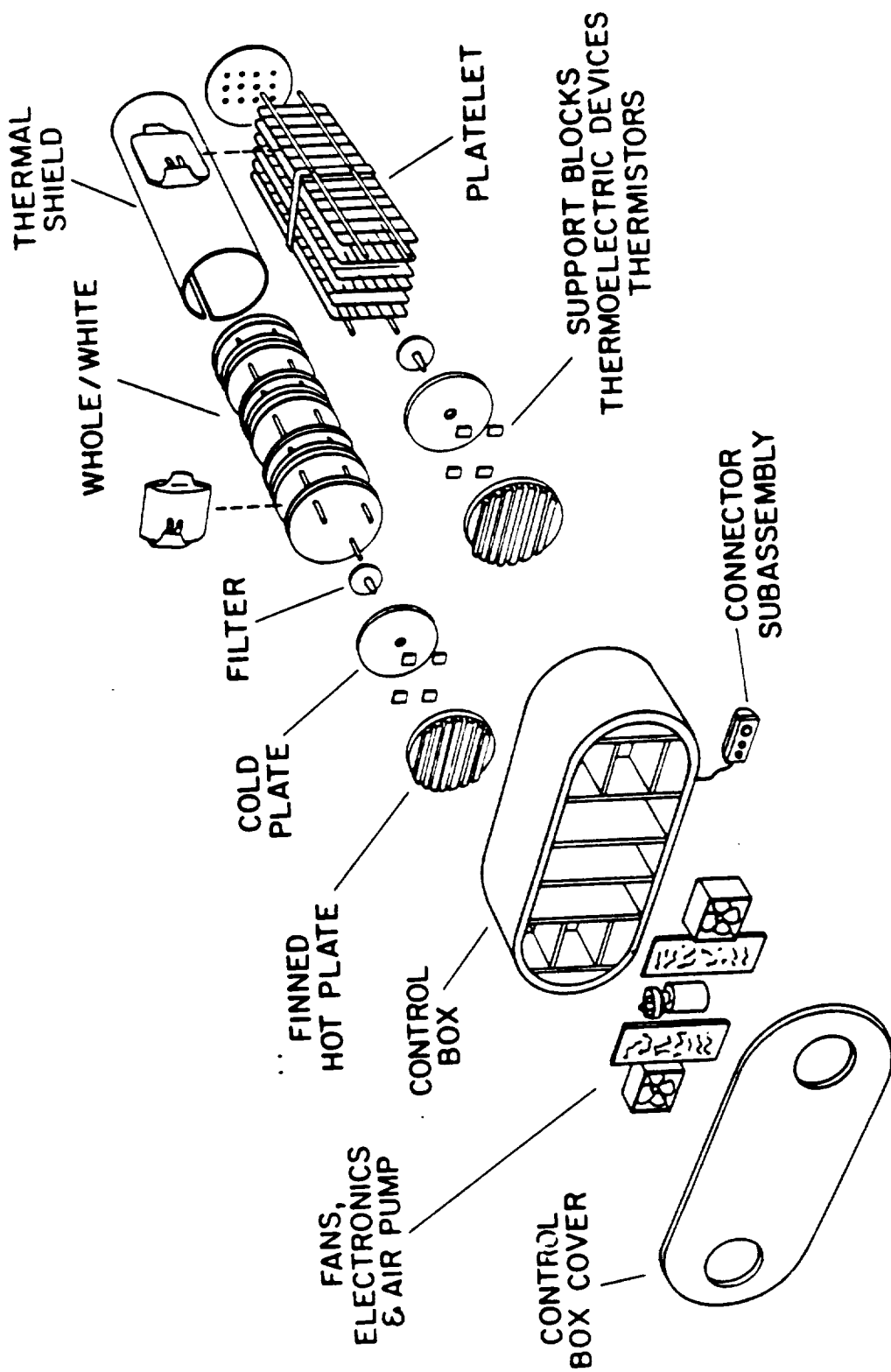


FIGURE 6-2 DETAILED EXPLODED VIEW OF THE COLD/WARM MODULE

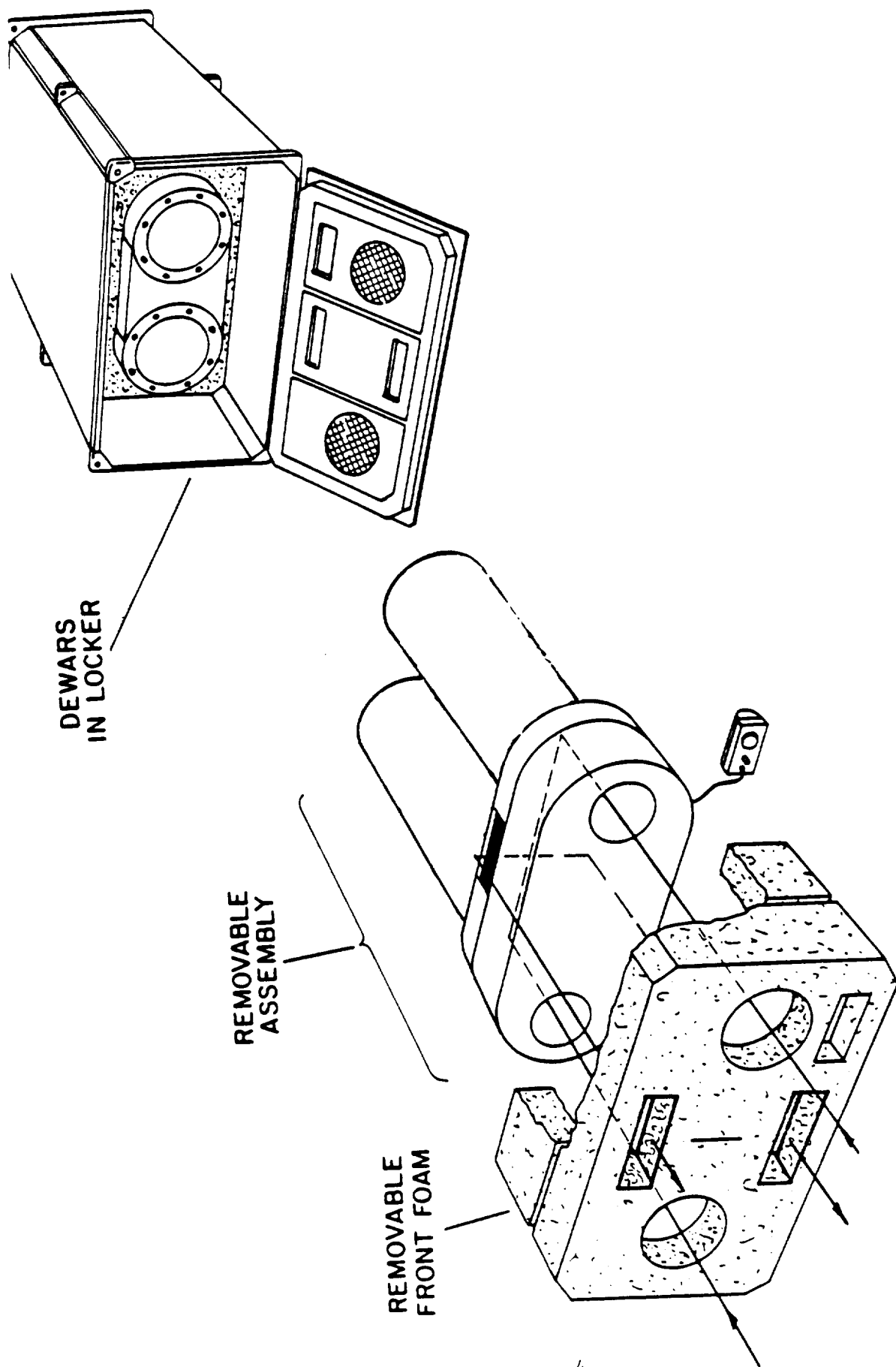


FIGURE 6-3 OVERALL VIEW OF THE COLD/COLD MODULE

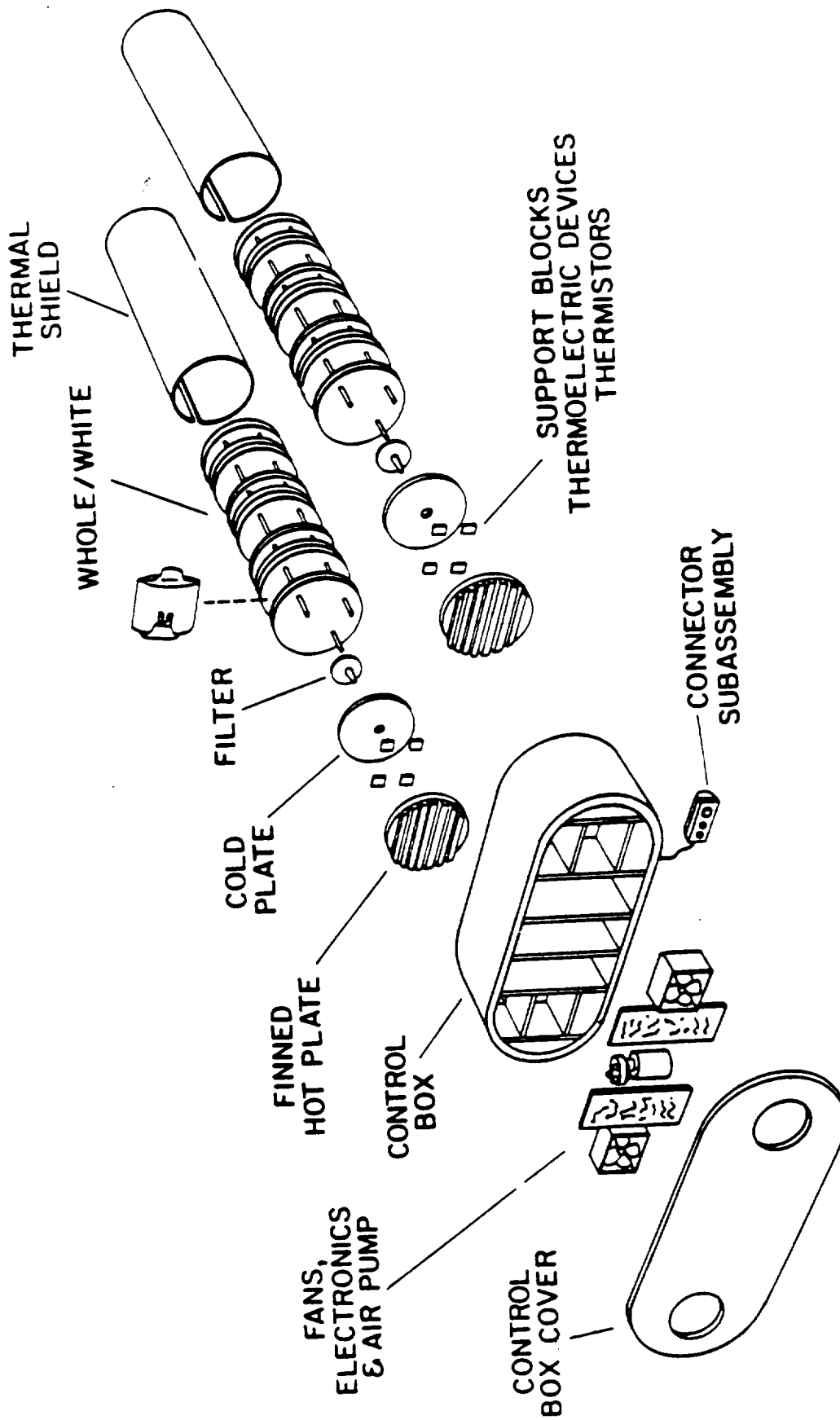


FIGURE 6-4 A DETAILED EXPLODED VIEW OF THE COLD/COLD MODULE

best measured at the exit of the air tube which terminates inside each dewar at its closed end. The following procedure is required to measure the air flow rate:

- 6.1.1 Disassemble control box from flanges of two dewars by removing eight, allen head screws around the perimeter of each of the two dewars.
- 6.1.2 Lift control box assembly and bag support structures from dewar assembly and lay on its side on a piece of soft material, e.g., a silicone rubber mat.
- 6.1.3 Remove the open end of the short horizontal section of TFE air tubing at the bottom of each dewar from inside the thermal shield or bag support structure.
- 6.1.4 Attach a calibrated (air) orifice or similar device to the exit of each of the two exposed ends of the air tubing. An air filter identical to the one in the cold plate can be used.
- 6.1.5 Install an inclined water manometer, or a similar pressure differential measuring device, to read the pressure drop across the orifice or similar device. The air flow pressure drop characteristics of the orifice should be approximately 0.1 inch of water at 100 ml/min air flow.
- 6.1.6 Apply 28 v dc power to the module via the connector on the electrical subpanel and measure the flow rate of air exiting each air tube.
- 6.1.7 If adjustments are required to the air flow rate, turn off the power to the module, and remove the top cover of the control box.
- 6.1.8 Adjustment to the air flow to both dewars, simultaneously, is achieved by changing the setting of the teflon valve located in the center compartment of the control box. This valve setting controls the fraction of the air output from the pump which flows to the two dewars. The remaining fraction of air flows to the electronic compartments of the control box as cooling air.
- 6.1.9 If the fraction of the air flow rate to one dewar has to be changed with respect to the air flow rate to the second dewar of the same assembly, install a small bore tube inside the appropriate TFE air tube at the bottom of the dewar. This additional flow restriction serves to divide the flow between the two dewars coming from a common source upstream. Table 6.1.9-1 summarizes the air flow rates set in all of the IBSE modules at the time of delivery of hardware to NASA.

TABLE 6.1.9-1 MEASURED AIR FLOW RATES TO IBSE DEWARS
 o OCTOBER 27, 1985

| | | | |
|----------|-------|----------------|----------------|
| S/N 0001 | (J/K) | COLD: 63 ML/M | WARM: 250 ML/M |
| S/N 0002 | (A/B) | COLD: 100 ML/M | COLD: 94 ML/M |
| S/N 0003 | (C/D) | COLD: 50 ML/M | WARM: 240 ML/M |
| S/N 0004 | (E/F) | COLD: 75 ML/M | WARM: 263 ML/M |
| S/N 0005 | (G/H) | COLD: 106 ML/M | COLD: 119 ML/M |

6.2 Thermal Performance Test

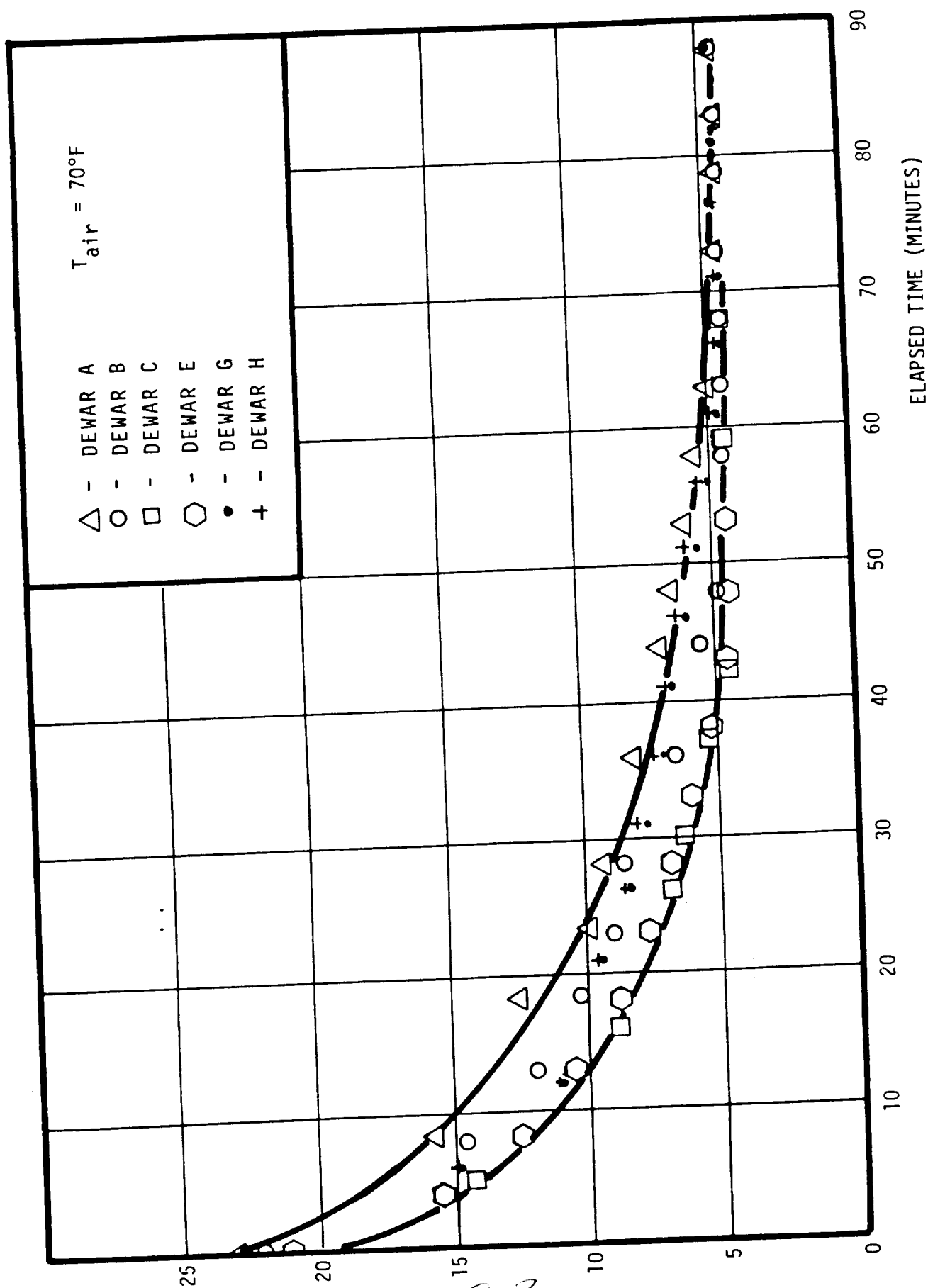
The thermal performance of the dewars can be measured from the exterior of the module without disassembly. There is a YSI model 44006 thermistor mounted on the cold plate of each dewar whose resistance can be read from test jacks on the front electrical subpanel of each module. The thermal performance test procedure using these test jacks is as follows:

- 6.2.1 Mount the IBSE module inside a block of foam similar to the Pyrell foam used in a middeck locker.
- 6.2.2 Electrically attach a resistance measuring meter to each of the two, white, test jacks on the front panel. The left, white, test jack is connected to the left dewar, etc. The third, blue, test jack, located below the two, white, test jacks as viewed from the front of the panel with the primary power connector to the right, is common to both thermistors.
- 6.2.3 Apply 28 v dc power to the module.
- 6.2.4 Record the transient temperature of each cold plate during cooldown.
- 6.2.5 Figure 6.2.5-1 is a plot of the transient cooldown temperature of each of the cold plates in the seven cold dewars ($5 \pm 1^{\circ}\text{C}$ steady state) recorded just prior to the time of delivery of the flight hardware to NASA with an ambient air temperature of approximately 70°F .
- 6.2.6 If the transient temperatures of a dewar does not approximately correspond with its previously measured values (see Figure 6.2.5-1), then initiate a detailed inspection and testing of all hardware and electronic subassemblies using engineering and electrical drawings.

7.0 Fault Analysis

The primary parameters to measure for an operating IBSE module are the temperatures of the cold plates inside the dewar (see Figure 6.2.5-1) and the current draw of the locker. With an ambient air temperature of approximately 70°F , the initial current draw of a cold/cold module (at 28 v dc) is approximately 3.5 amperes. The steady state current draw of the same locker for the same air temperature is approximately 1.5 amperes. The initial current draw of a cold/warm module is approximately 3.5 amperes and its steady state value is approximately 1 ampere. The current being drawn by a cold/warm module may show a 5 - 10 second cyclical variation of approximately .1 ampere due to the controller for the warm dewar periodically shutting off. If

FIGURE b.2.5-1
TRANSIENT COOLDOWN OF FIVE COLD IBSE DEWARs - OCTOBER 27, 1985



measured current values are significantly different from the values plotted in Figure 6.2.5-1, a detailed inspection of the electrical subsystem should be initiated.

There are three thermostats located inside the control box which will open if local, internal temperatures exceed 118°F. The thermostats are in series and will shut off all power to the module except to the two air cooling fans. There is one additional thermostat on each cooling fan to turn off power to that fan if its temperature exceeds 118°F.

There are three fuses inside the control box which, if blown, will shut off power to one of the two electronic temperature controllers (3a) or to the entire module (7a).

8.0 Operating Instruction

With the control box bolted to the dewar assembly, and a block of foam surrounding the assembly, the only remaining action to be taken by a user to initiate operation of the module is to apply 28 v dc power to the electrical connector on the front of the electrical subpanel. The application of 28 v dc will cause the green LED on the front of the locker to light. A blown fuse inside the locker does not turn the LED off. In a 0-g environment, the air flow flag over the exit air slot or grille will flutter to show that the cooling air fans are operating and, therefore, the module is receiving power.

After operating a cold dewar for an extended period of time, the cold plate/bag support structure should be removed from the dewar assembly and any condensed water removed. A jet of pressurized air can be used to blow out any moisture which has accumulated around the thermoelectric devices which are located in the gap between the hot and cold plates of the dewar lid assembly.

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THE CENTER FOR BLOOD RESEARCH

800 Huntington Avenue, Boston, Massachusetts 02115 (617) 731-6470

November 19, 1985

Mr. William Paton/LSSM
CS-SED-4
NASA
JFK Space Center
Florida 33899

RE: NAS 9-17222/IBSE

Dear Bill:

Enclosed please find a copy of the DD 1149 form with equipment and supply list attached.

For your information we also enclose an itemized bill-of-lading made out by the transportation company, Daley & Wanzer (form #1190).

The equipment will be leaving Boston on Friday, November 22nd, and will be delivered between 8 am and noon on Friday, December 6th.

If there are any changes that are required we are able to reach the driver in transit on a daily basis.

Please be advised that there are four IEC centrifuges that weigh approximately 1100 lbs. each and are palletized for fork-lift unloading at KSC.

Very truly yours,



Richard L. Korn
Executive Vice President

RLK:rar
Encl.

cc: E. Michel/EX4
✓D. Surgenor, CBR
F. Lionetti, CBR
C. Kelliher, ONR

RECEIVED

NOV 20 1985

PRESIDENT'S OFFICE

SHIPPING CONTAINER TALLY 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 37 38 39 40 41 42 43 44 45 46 47 48 49 50

REQUISITION AND INVOICE/SHIPPING DOCUMENT

1. FROM: ONRR/Boston Office Barnes Building
495 Summer Street, Boston, Massachusetts 02210-2109

2. TO: The Center for Blood Research
800 Huntington Avenue
Boston, MA 02115

3. SHIP TO - MARK FOR
Transportation Officer, NASA
c/o EG&G Florida, Inc.
Bldg. M-6-698
Kennedy Space Center, FL 32899

Deliver to: Jerry Moyer
Telephone: 305-853-7701
Hangler L, Cape Canaveral AFB
Mark for: Fabian J. Lionetti

4. APPROPRIATION AND SUBHEAD

5. REQUISITION DATE 19 Nov 1985

6. REQUISITION NUMBER NAS 9-17222-RQ-003

7. DATE MATERIAL REQUIRED

8. PRIORITY

9. AUTHORITY OR PURPOSE

10. SIGNATURE Edward Hertanen for

11. VOUCHER NUMBER AND DATE

12. DATE SHIPPED 19 November 1985

13. MODE OF SHIPMENT

14. BILL OF LADING NUMBER

15. AIR MOVEMENT DESIGNATOR OR PORT REFERENCE NO.

16. FEDERAL STOCK NUMBER, DESCRIPTION, AND CODING OF MATERIAL AND/OR SERVICES

17. SPECIAL HANDLING

18. TRANSPORTATION VIA MATS OR MATS CHARGEABLE TO

19. CONTAINERS RECEIVED AS NOTED

20. RECEIVER'S VOUCHER NO.

Contractor Furnished Equipment
see attached sheets: A = Damon/IEC
B = U. of Mass.
C = Children's Hospital
D = Center for Blood Research
E = Arthur D. Little

Requisition Distribution:
Copy No. Original - Consignee
1. Bill Paton
2. Richard Korn
3. ONR/C.F. Kelliher
4. NASA (ATTN: Ed Miche1/EX4)
5. ADL (ATTN: David Almgren)

Equipment is to be returned to
The Center for Blood Research
after completion of the experiments.

DD FORM 1 MAR 59 1149 (9-PT) 51 52 53 54 55 56 57 58 59 60 61 62 63 64 65 66 67 68 69 70 71 72 73 74 75 76 77 78 79 80 81 82 83 84 85 86 87 88 89 90 91 92 93 94 95 96 97 98 99 100

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OF POOR QUALITY

ORIGINAL

S/N 0102-LF-011-1801

REPLACES EDITION OF 1 MAY 58 WHICH MAY BE USED

Attachment A to DD 1149 19 Nov. 1985 Reg. # NAS 9-17222 RQ003

DAMON / IEC DIVISION

(p. 1)

15 Boxes - includes:

| | | |
|----|---------------------------------|----------|
| 5 | rotor assembly | (216) |
| 12 | nylon cushion conical | (315) |
| 12 | shield | (320) |
| 6 | trunnion ring | (326) |
| 6 | trunnion ring | (354) |
| 24 | Shield | (356) |
| 2 | rotor \bar{c} tubes | (889) |
| 3 | rotor \bar{c} tubes & cap | (890) |
| 1 | rotor \bar{c} tubes | (897) |
| 2 | head | (921) |
| 1 | rotor \bar{c} motor & inst | (977) |
| 4 | cytology buckets | (1024) |
| 8 | metal sealing caps | (1580) |
| 1 | DPR 6000 200/200V 60Hz | (2257) * |
| 1 | auto clear tubes | (2278) |
| 2 | Centra 7R (115/60 cycle) | (2360) * |
| 5 | Centra 7 (115/60 cycle) | (2362) * |
| 1 | Case auto clear tube | (2816) |
| 1 | CS auto clear tubes | (2997) |
| 3 | M-25 | (3600) * |
| 3 | Micro-MB 120V/50/60 Hz | (3615) * |
| 8 | Centrac adapters 7 place - blk | (5707) |
| 16 | Centrac adapter 19 place red | (5719) |
| 12 | Centrac adapter 37 place yellow | (5737) |
| 4 | holder cyto bucket | (5799) |
| 4 | adapter | (7224) |

NASA IBSE IMMUNOLOGY: UMASS MED CTR.

(p. 2)

LIST OF ITEMS TO BE SHIPPED FROM C.B.R.

| BOX 1: | Description | Qty. | |
|--------|--------------------------------------|------|--------------------|
| | Assorted plastic pipet tips | 2000 | |
| | 3 channel electronic timer | 1 | |
| | 20 ml syringe | 30 | |
| | Glass pasteur pipets, box of 100 | 3 | Approximate value: |
| | Vortex mixer | 2 | \$500.00 |
| | Glass 500ml vol. glask | 1 | |
| | 50 ml grad. centrifuge tube with cap | 50 | |
| | Parafilm, 4" x 250 ft. roll | 2 | |
| | | | |
| BOX 2: | | | |
| | Glycerol solution, 500 ml BTL | 2 | |
| | Aspirator pump | 3 | |
| | 10 ml red top tubes, 100/box | 3 | |
| | Wire test tube rack | 4 | |
| | Lab coat | 2 | |
| | 2000 ml plastic beaker | 2 | Approximate value: |
| | Magnetic stir bars | 6 | \$300.00 |
| | 30 ml plastic vials with cap | 50 | |
| | Ball point pens | 6 | |
| | Laboratory markers, assorted | 10 | |
| | Scissors | 1 | |
| | Hemostat | 2 | |
| | Paper clips, box | 1 | |
| | Tape, roll | 4 | |
| | | | |
| BOX 3: | | | |
| | 5 ml red top tube, 100/box | 3 | |
| | Plastic transfer pipets, 500/box | 3 | |
| | Plastic bottle 2 l | 1 | Approximate value: |
| | Plastic bottle 1 l | 2 | \$350.00 |
| | Plastic bottle 500 ml | 4 | |
| | Plastic bottle 250 ml | 2 | |
| | Plastic bottle, wash, 500 ml | 4 | |
| | Magnetic stirrer | 1 | |
| | | | |
| BOX 4: | | | |
| | Test tube mixer | 1 | Approximate value: |
| | Tape dispenser | 2 | \$290.00 |

Attachment B to DD 1149

11 NOV. 1985

Key# NAS 9-17222 -RQ-003

(P. 3)

BOX 5:

Description

Qty.

| | |
|--|--------------|
| 20 L plastic carboy | 1 |
| Plastic grad. cylinder, 2L | 1 |
| Plastic grad. cylinder, 1L | 1 |
| Plastic grad. cylinder, 500 ml | 1 |
| Plastic grad. cylinder, 100 ml | 1 |
| Notebook & paper | 2 |
| Plastic vol. flask 1L | 2 |
| 5% Bovine albumin P.B.S. | 500 ml |
| 3 containers, each with the following: | |

180g NaCl

22.44g Na₂HPO₄

6.712g NaH₂PO₄

20 NaN₃

45 containers, each with 1.5g Na₂EDTA

Approximate value:
\$160.00

Attachment C to DD1149 19 Nov. 1985 Reg# NAS 9-17220 RQ-003

(p.4)

Dr. Jacobson and Dr. Kewy - Children's Hosp.

- 10 boxes

Dr. Ausprunk - Children's Hosp

- 3 boxes

Dr. Scanlon

- 1 box

Attachment D to DD1149 19 Nov. 1985 Reg# NAS 7-17222-RA-003

R. Fabian Lionetti (White Cells) Center for Blood Research (p.5)

Box 1 :

- 1 case (144) Dilu. vials
- 1 case (96) sampling site couplers
- 4 boxes 10x75 mm glass tubes
- 5 pkg 10 ml disp. pipets
- 2 bx 18 G syringes
- 2 bx 1000 ul pipet tips (blue)
- 2 bx (24) 300 ml T-packs
- 1 bx diff stains
- 5 pks (125) 17 x 100 ml tubes & caps
- 1 bx 10 cc. syringes

x 2 :

- 3 pks 50 ml tubes (orange top)
- 3 pks 15 ml tubes (orange top)
- 1 bx (100) 1 cc syringes
- 1 bx parafilm
- 2 50 yellow tip pipets (20-200 ul)
- immersion oil
- 48 sidearm erlenmeyer flasks
- 2 pks flask cups
- 2 pks top stoppers
- 2 pks sidearm stoppers
- 2 bx transfer pipets 5 3/4"
- 2 nalgene 500 ml sterilization filter
- 250 scintillation vials.

Attachment D to DD1149 19 Nov. 1985 Reg # NAS 9-17222-RQ-003

Box 3: glass beakers (5 ranging from 10 ml \rightarrow 500 ml)
timer

500 ml glass graduated cylinder

dextran (100g)

(2) scintiverse II scintillation cocktail

biohazard autoclave bags

(1 bx) 50 cc. syring

(2 bx) Kimwipes

5 test tube racks

DMSO (1 vial)

20 μ l pipetman

chemical recipes (copies)

count-off solution

(1) vortex mixer

(1) HP 45 calculator

(1 pk - 125) 12 x 75 mm tubes

5 N H_2SO_4

(2) plastic funnels

1 ml centrifuge tube & caps

(2) 1000 μ l pipetman

(2) 200 μ l pipetman

(1) 100 - 1000 μ l pipetman

(1) 10 - 100 μ l pipetman

rubber aspiration tubing

gamma tubes & caps (> 130)

1 pr. hemostats

1 pr. scissors

chemicals for 1 l PBS

radioactive label tape

white, red, & blue label tape

2 bx microscope slides

2 bx cover slips

3 spatulas

marker pens

Attachment D to DD1149 19 Nov 1985 K4# NAS 7-1722 K9005

Box # JKR1 Platelets

(p.7)

Countoff 4l

Sodium Phosphate dibasic 500g

EGTA 100g

Sodium azide 4.3g

SDS 250g

PMSF .02m/Ethanol 100ml

Tris 500g

ACD 2l

Sodium meta periodate 100g

triton X-100 ~~600~~ 100ml

Nonidet-P40 100ml

Attachment D to D1149 19 Nov. 1985 Reg # NAS - 9-17222 KR 003

Box

JKKRZ

Platelets

(p.8)

Folins Reagents

① CuSO_4 1% 500ml

② 2% Na_2CO_3 1000ml

③ 2% Na/K tartate 500ml

NaOH 500ml I original safety packing

attach ment D to 1144 DD

19 Nov. 1945

Reg# NAS - 7-17222RQ003

Box # JKK123

Platelets

(P.9)

KCl 500g

NaCl 500g

Dextrose 500g

Sucrose 500g

1 Bottle of Folin's Reagent 500ml

Cable 8 mesh anhydrous 500g

* garland*

Attachment D to DD 1149 17 Nov. 1985 Reg # NAS 9A222 RQ003

Box # JKKR 4 Platelets
Recorder for Dual Channel Aggregometer

(p.10)

attachment D to 1144 DD 19 Nov 1985 Key# NAS 9 1722 RD 003

Box # JKKR5 Platelets
Dual channel aggregometer

(p.11)

Attachment D to 1149DD 19 Nov 1985 Reg# NAS 917222 RA 03

Box # JKKR 6 Platelets

(p.12)

2 Bottles Hydrogen Peroxide 100ml ea
in Original Safety Packing

Paraformaldehyde 500g

magnesium chloride 500g

Sodium phosphate monobasic 500g

EDTA 250g

glycerin 500ml

PUR gloves 1 Box large

1 Box small

latex gloves 1 Box medium small

" " large

Bme in Safety Packing 100ml

Parafilm 1 Box

1 stir bar Teflon

8 bags of 25 ~~ml~~ 10 ml Pipettes

10 bags of 25 5ml "

6 bags of " 1ml "

1 Box of " 1ml "

Attachment D to 1149 DD 19 Nov. 1985 Reg# NAS 9 17322RQ003

(p.13)

Box # JKK127 Platelets

Hcl Special Safety Packing 500ml

Coagulation assay Waterbath

1 Box Siliconized Pasteur Pipette

2 Boxes Disposable " "

500 Blue pipette tips

1000. Yellow " "

3 racks for 50cc test tubes

3 100ml. Graduate Cylinders

1 2L " "

1 1L " "

1 500ml " "

2 racks for 15 ml test tubes

1 thermometer for Water bath

6 500ml plastic beaters

2 1000ml " "

1 250ml " "

2 100 ml " "

Attachment D to 1149DD 19 Nov. 1985 Req# NAS 9 17322 RQ 013

Box # SKCR8 Platelets

(p.14)

100 15ml Orange TOP test tubes

375 13x100 Blue " " "

500 12x75 Blue " " "

100 Scintillation Vials

1 bag of Scintillation vial caps

12 Reagent Bottles

Attachment D to 1144 DD 15 Nov 1985 Reg # NWS 9 A222

10 003

Box # JKKR9

Platelet

200 scintillation vials + tops (7ml) (p.15)

2 duFuge racks

2 5ml Sarstedt tube (control) racks

2 rolls of green tape

2 " " white "

1 " " red "

1 " " orange "

3 test tube racks

1 ice bucket

1/2 Box of Fisher weigh paper

20 lg weigh boats

6 spatulas

1000 control Sarstedt tubes

250 red " caps

250 Blue " "

250 white " "

250 yellow " "

2 markers felt tip

2 Boxes km-wipe

Attachment D to 1144 DD 19 Nov. 1985 Reg# NAS 9 17222 RQ 003

3 boxes - Debbie Van Delt
Corning tubes etc.

(p. 15)

2 boxes - Curby Hirsts *

Hachment E to 1149 DD 19 Nov. 1985 Reg# NAS 9 1722200
003

(P. 18)

Arthur D. Little

5 boxes → ^{includes} 2 NASA emptied containers

CONTRACTOR OR CARRIER

HOUSEHOLD GOODS DESCRIPTIVE INVENTORY

DALEY & WANZER, INC.

AGENT

D+W

| | |
|-------------------------|--------------|
| PAGE NO. | NO. OF PAGES |
| CARRIER'S REFERENCE NO. | |
| CONTRACT OR GBL. NO. | |
| GOVT. SERVICE ORDER NO. | |
| VAN NUMBER | |

| | |
|----------------------------------|---------------------------|
| OWNER'S GRADE OR RATING AND NAME | Center for Blood Research |
| ORIGIN LOADING ADDRESS | 800 Huntington Ave Boston |
| DESTINATION | Kennedy Space Center |

| | | |
|--|--|--|
| DESCRIPTIVE SYMBOLS B/W - BLACK & WHITE TV C - COLOR TV CP - CARRIER PACKED PBO - PACKED BY OWNER CD - CARRIER DISASSEMBLED DBO - DISASSEMBLED BY OWNER PB - PROFESSIONAL BOOKS PE - PROFESSIONAL EQUIPMENT PP - PROFESSIONAL PAPERS | EXCEPTION SYMBOLS BE - BENT BR - BROKEN BU - BURNED CH - CHIPPED CU - CONTENTS & CONDITION UNKNOWN D - DENTED F - FADED G - GOUGED L - LOOSE M - MARRED MI - MILDEW MO - MOTHEATEN R - RUBBED RU - RUSTED SC - SCRATCHED SH - SHORT SO - SOILED T - TORN W - BADLY WORN Z - CRACKED | LOCATION SYMBOLS 1. ARM 2. BOTTOM 3. CORNER 4. FRONT 5. LEFT 6. LEGS 7. REAR 8. RIGHT 9. SIDE 10. TOP 11. VENEER 12. EDGE |
|--|--|--|

NOTE: THE OMISSION OF THESE SYMBOLS INDICATES GOOD CONDITION EXCEPT FOR NORMAL WEAR.

| ITEM NO. | CR. REF. | ARTICLES | CONDITION AT ORIGIN | EXCEPTIONS (IF ANY) AT DESTINATION |
|----------|----------|--------------------|---------------------|------------------------------------|
| 1 | | | | |
| 2 | | LARGE WOODEN CRATE | | P.B. BRC |
| 3 | | " " " | | " |
| 4 | | SMALL CARTON | | P.B. BRC |
| 5 | | LARGE OPEN | | " |
| 6 | | " " " | | " |
| 7 | | MEDIUM FLAT | | " |
| 8 | | LARGE | | " |
| 9 | | X-tra Large | | " |
| 10 | | LARGE | | " |
| 1 | | MEDIUM | | " |
| 2 | | SMALL | | " |
| 3 | | " | | " |
| 4 | | " | | " |
| 5 | | MEDIUM | | " |
| 6 | | 3-4 | | " |
| 7 | | MEDIUM WHITE | D+W CARTON | " |
| 8 | | " | | " |
| 9 | | " | | " |
| 10 | | SMALL | | " |
| 1 | | " | | " |
| 2 | | LARGE | | " |
| 3 | | LARGE FLAT | | " |
| 4 | | " | | " |
| 5 | | SMALL | | " |
| 6 | | 3-1 | | " |
| 7 | | LARGE | D+W CARTON | " |
| 8 | | " | | " |
| 9 | | " | | " |
| 10 | | 3-1 | D+W CARTON | " |
| 1 | | " | | " |
| 2 | | LARGE | | " |
| 3 | | " | | " |
| 4 | | " | | " |
| 5 | | " | | " |
| 6 | | SHORT THIN | | " |
| 7 | | LARGE | | " |
| 8 | | " TALL | | " |
| 9 | | " THIN | | " |
| 10 | | " | | " |

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| | |
|----------|--------------------|
| ITEM NO. | REMARKS/EXCEPTIONS |
| | |

WE HAVE CHECKED ALL THE ITEMS LISTED AND NUMBERED 1 TO 10 INCLUSIVE AND ACKNOWLEDGE THAT THIS IS A TRUE AND COMPLETE LIST OF THE GOODS TENDERED AND OF THE STATE OF THE GOODS RECEIVED.

WARNING BEFORE SIGNING CHECK SHIPMENT. COUNT ITEMS AND DESCRIBE LOSS OR DAMAGE IN SPACE ON THE RIGHT ABOVE

| | | | | | |
|-------------|--|----------|----------------|--|------|
| AT ORIGIN | CONTRACTOR, CARRIER OR AUTHORIZED AGENT (DRIVER) | DATE | AT DESTINATION | CONTRACTOR, CARRIER OR AUTHORIZED AGENT (DRIVER) | DATE |
| (SIGNATURE) | Andrew Thaburn | 11/19/65 | (SIGNATURE) | | |
| (SIGNATURE) | | | (SIGNATURE) | | |

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CONTRACTOR OR CARRIER

HOUSEHOLD GOODS DESCRIPTIVE INVENTORY

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| CONTRACT OR GBL. NO. | |
| GOVT. SERVICE ORDER NO. | |
| VAN NUMBER | |

DALEY & WANZER, INC.

AGENT

Dtu

OWNER'S GRADE OR RATING AND NAME

Center for Blood Research

CITY

STATE

ORIGIN LOADING ADDRESS

DESTINATION

Kennedy Space Center

DESCRIPTIVE SYMBOLS

B/W - BLACK & WHITE TV DBD - DISASSEMBLED BY OWNER
 C - COLOR TV PB - PROFESSIONAL BOOKS
 CP - CARRIER PACKED PE - PROFESSIONAL EQUIPMENT
 PBO - PACKED BY OWNER PP - PROFESSIONAL PAPERS
 CD - CARRIER DISASSEMBLED

BE - BENT
 BR - BROKEN
 BU - BURNED
 CH - CHIPPED
 CU - CONTENTS & CON-
 DITION UNKNOWN

EXCEPTION SYMBOLS

D - DENTED M - MARRED
 F - FADED MI - MILDewed
 G - GOUGED MO - MOTH EATEN
 L - LOOSE R - RUBBED
 RU - RUSTED

SC - SCRATCHED
 SH - SHORT
 SO - SOILED
 T - TORN
 W - BADLY WORN
 Z - CRACKED

LOCATION SYMBOLS

1. ARM 7. REAR
 2. BOTTOM 8. RIGHT
 3. CORNER 9. SIDE
 4. FRONT 10. TOP
 5. LEFT 11. VEEKEER
 6. LEGS 12. EDGE

NOTE: THE OMISSION OF THESE SYMBOLS INDICATES GOOD CONDITION EXCEPT FOR NORMAL WEAR.

| ITEM NO. | CR. REF. | ARTICLES | CONDITION AT ORIGIN | EXCEPTIONS (IF ANY) AT DESTINATION |
|----------|----------|--------------------|---------------------|------------------------------------|
| 1 | | X-tra Large Carton | | P.B. ARC |
| 2 | | " " " | | " |
| 3 | | " " " | Very Fragile | " |
| 4 | | " " " | " " | " |
| 5 | | " " " | " " | " |
| 6 | | Large Thin | | " |
| 7 | | " " " | | " |
| 8 | | 3-1 | Dtu Carton | " |
| 9 | | " " " | " " | " |
| 10 | | " " " | " " | " |
| 11 | | Large | | " |
| 12 | | " " " | | " |
| 13 | | " " " | | " |
| 14 | | " " " | | " |
| 15 | | " " " | | " |
| 16 | | " WHITE | | " |
| 17 | | " " " | | " |
| 18 | | " " " | | " |
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ITEM NO. REMARKS/EXCEPTIONS

WE HAVE CHECKED ALL THE ITEMS LISTED AND NUMBERED 1 TO 50 INCLUSIVE AND ACKNOWLEDGE THAT THIS IS A TRUE AND COMPLETE LIST OF THE GOODS TENDERED AND OF THE STATE OF THE GOODS RECEIVED

BEFORE SIGNING CHECK SHIPMENT, COUNT ITEMS AND DESCRIBE LOSS OR DAMAGE IN SPACE ON THE RIGHT ABOVE

| | | | | | |
|--|--|----------|--|--|------|
| CONTRACTOR, CARRIER OR AUTHORIZED AGENT (DRIVER) | | DATE | CONTRACTOR, CARRIER OR AUTHORIZED AGENT (DRIVER) | | DATE |
| (SIGNATURE) Andrew Wanzer | | 11/18/80 | (SIGNATURE) | | DATE |
| OWNER OR AUTHORIZED AGENT | | DATE | OWNER OR AUTHORIZED AGENT | | DATE |
| (SIGNATURE) | | | (SIGNATURE) | | |

FORM 1180 REV. '71

MILBURN PRINTING, INC. 138 SCHMITT BLVD. FARMINGDALE, N. Y. 11735

TABLE I
SUMMARY OF MAIN IBSE ACTIVITY

| | PLAN K* | PLAN KE* | PLAN E* |
|---|------------|--|---|
| Primary Landing Site | KSC | -- | Edward |
| Secondary Landing Site | -- | Edward | -- |
| Location of Lab & Investigators | KSC | KSC | Boston |
| Flight Hardware at the time of launch | KSC | KSC | KSC |
| Ground samples (Zero time and Control) | KSC | KSC | Boston |
| Loading of the Dewars | KSC | KSC | KSC |
| Transportation of ground control after launch | Within KSC | Within KSC | From KSC to Boston in dewars via air-conditioned van |
| Transportation of flight sample after launch | Within KSC | From Edward to KSC via a jet arranged by IBSE team | From Edward to Boston via a jet arranged by IBSE team |

Plan K: KSC landing
 Plan KE: Edward landing site with KSC as primary landing site
 Plan E: Edward as the primary landing site

TABLE II

PRE-LAUNCH ACTIVITY

| ACTIVITY CODE | PLAN* INVOLVEMENT | DESCRIPTION | TARGET DATE & PERIOD | LOCATION | PERSONNEL |
|------------------|----------------------|---|----------------------------|------------------|-----------|
| H-1 | K, KE, E | Acceptance of Flight Hardware | | Boston | |
| H-2 | K, KE, E | Storage of Flight Hardware | | ADL Boston | |
| H-3 | K, KE, E | Transportation of 5 units of hardware to KSC | | Boston to KSC | |
| H-4 | K, KE, E | Receiving and Testing of hardware | | KSC | |
| L-1 | K, KE | Inventory of equipment and labwares to be shipped to KSC | | CBR Boston | KM |
| L-2 | K, KE | Packing of equipment and labwares | | CBR Boston | |
| L-3 | K, KE | Shipping of equipment and and labwares to KSC | | Boston to KSC | |

* Plan K: KSC landing
 Plan KE: Edward landing with KSC as the primary landing site
 Plan E: Edward landing with Edward as the primary landing site

| ACTIVITY CODE | PLAN* INVOLVEMENT | DESCRIPTION | TARGET DATE & PERIOD | LOCATION | PERSONNEL |
|------------------|----------------------|--|----------------------------|---------------|-----------|
| L-4 | K, KE | Receiving of equipment and labwares | | KSC | |
| L-5 | K, KE | Setting up IBSE Lab | | KSC | |
| L-6 | K, KE | Check-out equipment and labwares | | KSC | |
| P-1 | K, KE | List of Personnel and clearance | | Boston KSC | |
| P-2 | K, KE | Arrangement for transporta- tion of personnel (Air and Ground) | | Boston KSC | |
| P-3 | K, KE | Room and Board | | KSC | |
| P-4 | K, KE | Communication | | Boston KSC | |

| ACTIVITY CODE | PLAN* INVOLVEMENT | DESCRIPTION | TARGET DATE & PERIOD | LOCATION | PERSONNEL |
|------------------|----------------------|--|----------------------------|----------------------|--|
| B-1 | K, KE, E | Collection of 36 units of whole blood | | CFBB Orlando | CFBB Personnel DV & MJ |
| B-2 | K, KE, E | Component Preparations | | CFBB | FL and Crew DV & MJ CFBB Personnel |
| B-3 | K, KE, E | Pooling Blood Samples | | CFBB | DV, MJ, BP |
| B-4 | K, KE, E | Allocating Blood Samples | | CFBB | DV, MJ, BP |
| B-5 | K, KE, E | Transportation of 56 units of Blood samples to KSC | | Orlando to KSC | MJ |
| B-6 | K, KE | Transportation to Zero Time Sample | | Orlando to KSC | MJ |
| B-7 | E | Transportation of Zero Time Sample | | Orlando to Boston | BP |

| ACTIVITY CODE | PLAN* INVOLVEMENT | DESCRIPTION | TARGET DATE & PERIOD | LOCATION | PERSONNEL |
|------------------|----------------------|---|----------------------------|----------|--------------------------------------|
| D-1 | K, KE, E | Preloading preparation of the Dewars | | KSC | EB, DA, WC JY, RI |
| D-2 | K, KE, E | Preloading storage of Blood Samples | | KSC | NJ, FC |
| D-3 | K, KE | Coding and distributing of zero time samples | | KSC | DVP, BP |
| D-4 | K, KE, E | Loading Blood bags into the dewars | | KSC | EB, DA, WC JY, RI, NJ, DVP, BP |
| D-5 | K, KE, E | Close off the dewars | | KSC | |
| D-6 | K, KE, E | Transfer flight lockers with test samples to NASA personnel | | KSC | |
| D-7 | K, KE, E | Install IRSE dewars/lockers in the space shuttle | | KSC | NASA personnel |

| ACTIVITY CODE | PLANET INVOLVEMENT | DESCRIPTION | TARGET DATE & PERIOD | LOCATION | PERSONNEL |
|------------------|-----------------------|---|----------------------------|---------------|----------------|
| D-8 | K, KE, E | Connecting and checking power supply to the dewars | | KSC | NASA personnel |
| D-9 | K, KE | Transport loaded ground lockers to the storage area | | KSC | |
| D-10 | K, KE | Connect and check power supply to the ground/dewars lockers | | KSC | |
| D-11 | K, KE | Storage of ground lockers | | KSC | |
| E-1 | E | Coding and distributing zero time samples | | Boston | DVP, BP |
| E-2 | E | Transport loaded ground lockers with power supply connected to Boston | | KSC to Boston | |
| E-3 | E | Storage of ground lockers in Boston | | CBR Boston | |

| ACTIVITY CODE | PLAN* INVOLVEMENT | DESCRIPTION | TARGET DATE & PERIOD | LOCATION | PERSONNEL |
|------------------|----------------------|---|----------------------------|--------------------------|-----------|
| E-4 | E | List of location of laboratories | | Boston | |
| E-5 | E | Request for special and additional equipment | | Boston | |
| E-6 | E | Distribution of equipment | | Boston | |
| E-7 | E | Check out equipment | | Boston | |
| E-8 | E | List of Personnel and clearance | | Boston | |
| E-9 | E | Arrangement for Transportation (Air and Ground) | | Orlando KSC Boston | |
| E-10 | E | Rooms and board | | Orlando KSC | |
| E-11 | E | Communication | | Orlando KSC Boston | |

TABLE IV
POST LANDING ACTIVITY

| ACTIVITY CODE | PLAN INVOLVEMENT | DESCRIPTION | TARGET DATE & PERIOD | LOCATION | PERSONNEL |
|------------------|---------------------|--|----------------------------|--|---------------------------|
| PL-1 | K, KE, E | Dewar/locker operational check prior to removal from the Shuttle | | KSC (Plan K) Edward (Plan KE or E) | NASA Personnel |
| PL-2 | K, KE, E | Remove the dewars from the Shuttle | | KSC (Plan K) Edward (Plan KE or E) | NASA Personnel |
| PL-3 | K, KE, E | Connecting to Battery power supply | | KSC (K) Edward (KE or E) | NASA Personnel |
| PL-4 | K | Transport Flight lockers to IBSE lab and turn over to IBSE team | | KSC | NASA IBSE Personnel |
| PL-5 | K, KE, E | Transport ground lockers to IBSE Lab | | KSC (Plan K & KE) Boston (Plan E) | |
| PL-6 | K, KE, E | Locker operational check prior to opening | | KSC (Plan K & KE) Boston (Plan E) | WC |

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| ACTIVITY CODE | PLAN INVOLVEMENT | DESCRIPTION | TARGET DATE & PERIOD | LOCATION | PERSONNEL |
|------------------|---------------------|--|----------------------------|--|---------------------------|
| PL-7 | K, KE, E | Opening the dewars lifting the content photography | | KSC (Plan K & KE) Boston (Plan E) | Hoist team members |
| PL-8 | K, KE, E | Remove Blood bags and fill out check list | | KSC (Plan K & KE) Boston (Plan E) | |
| PL-9 | K, KE, E | Coding and distribution of Blood samples | | KSC (Plan K & KE) Boston (Plan E) | |
| PL-10 | K, KE, E | Testing and Assay | | KSC & Boston (Plan K & KE) Boston (Plan E) | IBSE Investiga tors |
| PL-11 | K, KE, E | Hoist Team on position | | KSC (Plan K & KE) Boston (Plan E) | |
| PL-12 | K, KE, E | Coding and distribution team in position | | KSC (Plan K & Boston (Plan E) | |
| PL-13 | K, KE, E | Investigator team in position | | KSC (Plan K & KE) Boston (Plan E) | |

| ACTIVITY CODE | PLAN INVOLVEMENT | DESCRIPTION | TARGET DATE & PERIOD | LOCATION | PERSONNEL |
|------------------|---------------------|--|----------------------------|----------------------------------|-------------------|
| PL-14 | KE, E | Transport flight lockers to MJV Field Airport & turn over to INSE team | | Edward Air Base | NASA Personnel |
| PL-15 | KE, E | Locker Operational Check | | Edward | |
| PL-16 | KE, E | Secure lockers and batteries into the transporting jet | | Edward | |
| PL-17 | KE, E | Fly sample locker to INSE lab location | | KSC (Plan KE) Boston (Plan E) | |
| PL-18 | KE, E | Remove lockers and batteries from the jet | | KSC (Plan KE) Boston (Plan E) | |
| PL-19 | KE, E | Transport lockers and batteries to INSE lab | | KSC (Plan KE) Boston (Plan E) | |

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△ Arthur D. Little, Inc.

October 15, 1985

McDonnell Douglas Technical
Services, Inc.
16441 Space Center Boulevard
Houston, TX 77058

Attention: Mr. Stephen Gotch
Mail Code D3

Subject: Power Dissipation of the Initial Blood Storage
Experiment

Gentlemen: ADL Reference 53282-51

The following steady-state power dissipation data for the Initial Blood Storage Experiment (IBSE) is based on test data recorded during the period of time from October 2, 1985 to October 9, 1985, with a 28V dc power source.

| | <u>Power</u> |
|--|--------------|
| 1.0 Air pressure: 14.7 psia | |
| 1.1 T _{air} - 65°F | |
| C/C Module (4215-1400) | 20W |
| C/W Module (4215-1200) | 18W |
| 1.2 T _{air} - 75°F | |
| C/C Module (4215-1400) | 31W |
| C/W Module (4215-1200) | 25W |
| 1.3 T _{air} - 80°F | |
| C/C Module (4215-1400) | 50W |
| C/W Module (4215-1200) | 52W |
| 1.4 T _{air} - 90°F (max. power dissipation capability of Modules @ 28 Vdc) | |
| C/C Module (4215-1400) | 81W |
| C/W Module (4215-1200) | 70W |

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Arthur D. Little, Inc.

McDonnell Douglas Technical
Services, Inc.
Attention: Mr. Stephen Gotch

2.0 Air Pressure: 10.2 psia

| | | |
|-----|--|------|
| 2.1 | $T_{\text{air}} = 65^{\circ}\text{F}$ | |
| | C/C Module (4215-1400) | 21W |
| | C/W Module (4215-1200) | 22W |
| 2.2 | $T_{\text{air}} = -75^{\circ}\text{F}$ | |
| | C/C Module (4215-1400) | 34W |
| | C/W Module (4215-1200) | 44W |
| 2.3 | $T_{\text{air}} = 80^{\circ}\text{F}$ | |
| | C/C Module (4215-1400) | 49W |
| | C/W Module (4215-1200) | 54W. |

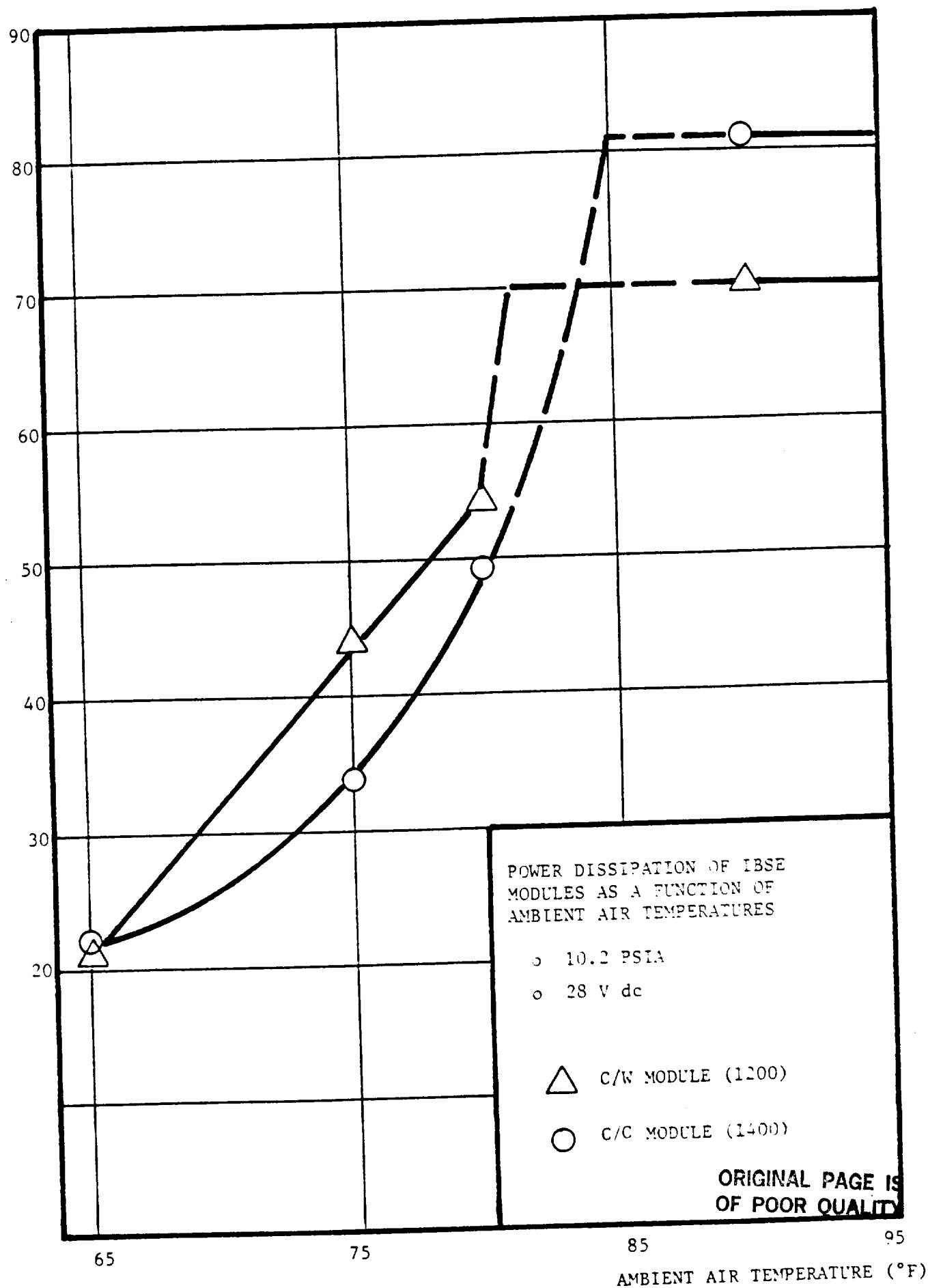
If you have any questions, please feel free to call me.

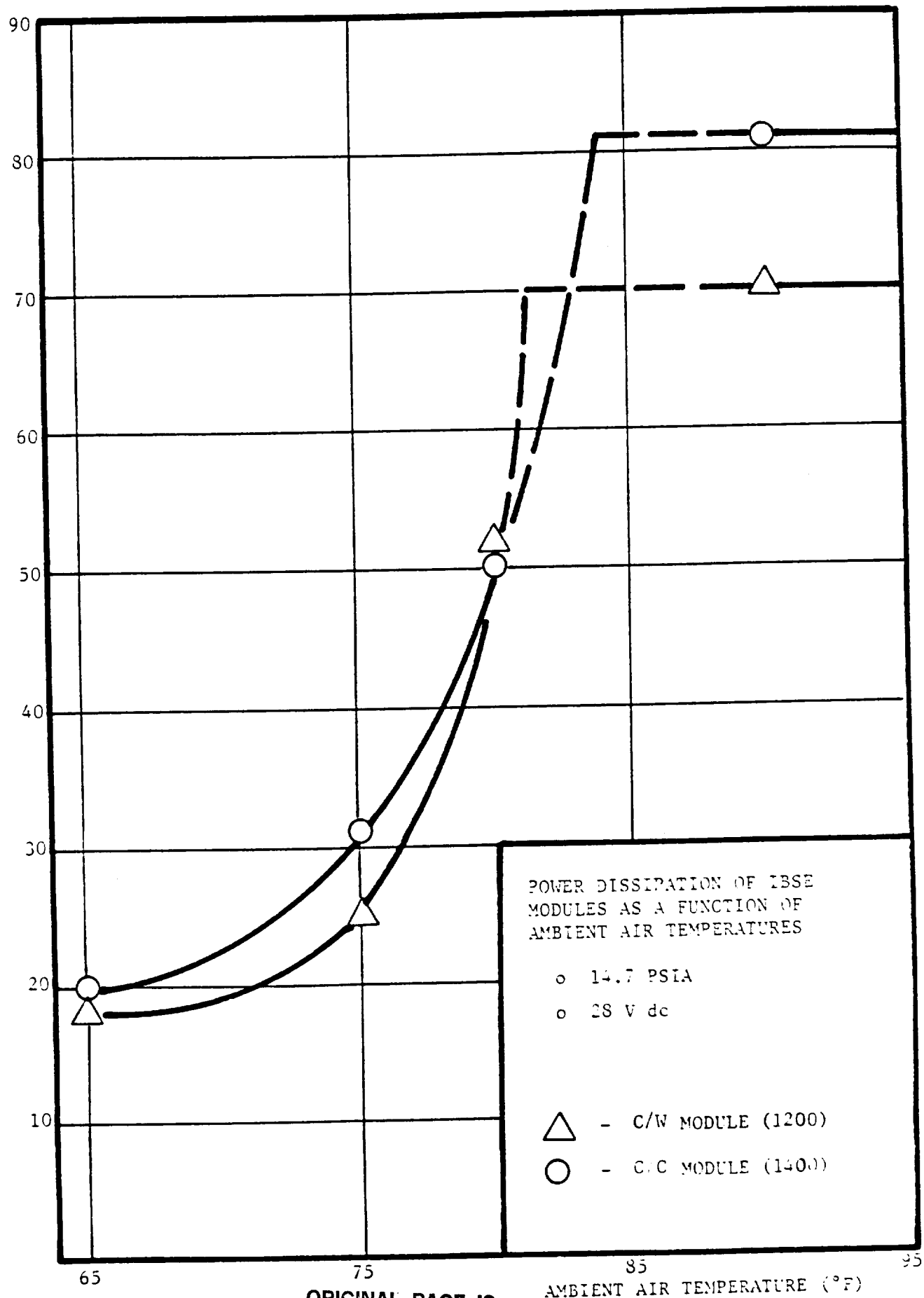
Sincerely,

David W. Almgren

David W. Almgren
IBSE Engineering Manager

dwa:ln





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Arthur D. Little, Inc.

October 10, 1985

To: D. MacN. Surgenor
IBSE Principal Investigator

From: D. Almgren
IBSE Engineering Manager

Subject: Quick Look Report for IBSE Thermal Performance Tests

During the one week period from October 2, 1985 to October 9, 1985, a cold/cold IBSE module (S/N 0005) and a cold/warm IBSE module (S/N 0001) were tested over a range of temperatures at two pressures: 14.7 and 10.2 psia. This Quick Look Report represents a summary of the steady state raw data as recorded during the tests.

I. Steady State Condition No. 1 $T_{\text{air}} = 65^{\circ}\text{F}$ Pressure = 14.7 psia

Date: October 3, 1985 @ 1200

C/C Module: cold plate No. 1: 4.4°C
cold plate No. 2: 4.3°C
T: 1.3°C (top to bottom)
 T_{air} : 6.1°C (entering at bottom)
Power: 20W

C/W Module: cold plate (C): 4.2°C
cold plate (W): 21.0°C
Power: 18W

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London
Los Angeles

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Mexico City
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Paris

San Francisco
São Paulo
Singapore
Tokyo

Toronto
Washington
Wiesbaden

October 10, 1985 Page 2

II. Steady State Condition No. 2 $T_{\text{air}} = 75^{\circ}\text{F}$ Pressure = 14.7 psia

Date: October 4, 1985 @ 0840

C/C Module: cold plate No. 1: 4.5°C
cold plate No. 2: 4.3°C
T: 1.4°C (top to bottom)
 T_{air} : 6.4°C (entering at bottom)
Power: 31W

C/W Module: cold plate (C): 4.4°C
cold plate (W): 21.3°C
Power: 25W

III: Steady State Condition No. 3 $T_{\text{air}} = 90^{\circ}\text{F}$ Pressure = 14.7 psia

Date: October 5, 1985 @ 1030

C/C Module: cold plate No. 1: 9.6°C
cold plate No. 2: 8.7°C
T: 2.1°C (top to bottom)
 T_{air} : 11.7°C (entering at bottom)
Power: 81W

C/W Module: cold plate (C): 10.2°C
cold plate (W): 24.7°C
Power: 70W

IV. Steady State Condition No. 4 $T_{\text{air}} = 80^{\circ}\text{F}$ Pressure = 14.7 psia

Date: October 7, 1985 @ 1430

C/C Module: cold plate No. 1: 4.6°C
cold plate No. 2: 4.4°C
T: 1.9°C (top to bottom)
 T_{air} : 7.2°C (entering at bottom)
Power: 50W

C/W Module: cold plate (C): 5.6°C
cold plate (W): 21.5°C
Power: 52W

October 10, 1985 Page 3

V. Steady State Condition No. 5 $T_{\text{air}} = 80^{\circ}\text{F}$ Pressure = 10.2 psia

Date: October 8, 1985 @ 0900

C/C Module: cold plate No. 1: 4.6°C
cold plate No. 2: 4.4°C
T: 1.7°C (top to bottom)
 T_{air} : 7°C (entering at bottom)
Power: 49W

C/W Module: cold plate (C): 6.4°C
cold plate (W): 21.5°C
Power: 54W

VI. Steady State Condition No. 6 $T_{\text{air}} = 75^{\circ}\text{F}$ Pressure = 10.2 psia

Date: October 8, 1985 @ 1330

C/C Module: cold plate No. 1: 4.5°C
cold plate No. 2: 4.3°C
T: 1.6°C (top to bottom)
 T_{air} : 6.6°C (entering at bottom)
Power: 34W

C/W Module: cold plate (C): 4.5°C
cold plate (W): 21.5°C
Power: 44W

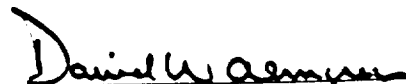
VII. Steady State Condition No. 7 $T_{\text{air}} = 65^{\circ}\text{F}$ Pressure = 10.2 psia

Date: October 9, 1985 @ 1300

C/C Module: cold plate No. 1: 4.4°C
cold plate No. 2: 4.2°C
T: 1.4°C (top to bottom)
 T_{air} : 6.3°C (entering at bottom)
Power: 22W

C/W Module: cold plate (C): 4.4°C
cold plate (W): 21.5°C
Power: 21W

Cc: R. Berthiaume
K. Csigi
W. Curby
P. Glaser


David W. Almgren

IBSE Data Analysis

Donald Blevins, Ph.D.
Nan Laird, Ph.D.

January 15, 1987

RECEIVED

JAN 16 1987

PRESIDENT'S OFFICE

DATA ANALYSIS

Experimental design:

Each of three different blood components, red cells, white cells and platelets were subjected to microgravity aboard the space shuttle. Each were stored in three different types of storage bags, PVC DEHP, PVC TOTM and Polyolefin and placed in labeled Dewar flasks. Controls matched both for type of storage bag and Dewar position remained on earth.

Upon return to earth, the contents of the bags as well as the contents of the control bags were distributed in randomly numbered samples to individual investigators. The investigators had no knowledge of which were the control samples or of the different storage conditions of the samples. A total of 74 different outcome measurements were reported to us for statistical analysis.

Method of analysis:

The data received from the investigators was listed only by coded sample number. The first step was to match the coded samples with the original bag numbers to determine the information on bag type, orbital status, and storage location. The method of analysis used was analysis of variance, using type of bag and orbital status as the main factors. Most data sets included either 18 or 20 samples, hence allowed investigation of a possible interaction effect as well. The analysis tool used was SAS (Statistical Analysis System, release 82.4, SAS Institute, Cary, NC). For those data sets with fully balanced factors, the SAS ANOVA procedure was used, while for the others the GLM (General Linear Models) procedure was used.

One of the bags (bag number 56) was found to have developed a leak at some time before the samples were taken for distribution to investigators. For experiments involving that bag, the statistical analysis was redone with it omitted.

Results:

The results of the statistical analysis are summarized in the following. The data are listed alphabetically by the name of the principal investigator - often in multiple tables if many measurements were done by the same investigator. The first column of each table is the sample number as given to the investigator, the second number is the number of the bag from which the sample was taken, the third column is the label of the Dewar flask in which the bag was stored. Flasks a to d were on the orbiter while e to h remained on earth. In terms of storage configuration, a and e, b and f, c and g, d and h occupied corresponding positions. The fourth column of the table gives the orbital status; 1 indicates orbit and 0 indicates a control. The

fifth column of the table indicates the type of bag used, d is PVC DEHP, p is Polyolefin, and t is PVC TOTM. The remaining column(s) contain the reported measurements. Following this is an explanation of the units of the measurement and a table which reports the mean of the measurement and the standard error of the mean (measurement of the accuracy of reported mean) for each combination of bag type and orbital and control samples. In addition, the overall means by bag type and earth/orbit status are reported in the column and row margins of the table. If the analysis of variance indicated statistical significance for one or more factors, the corresponding p-value is reported. If no such notation occurs, the factor was not significant (at the 5% level).

For experiments involving samples from bag number 56 as discussed above, the results of the analysis omitting that sample are reported in the same format, immediately following the original results.

Ausprunk - Transmission Electron Microscopy Data - Red Cells

| Sample | Bag | Dewar | Orbit=1 | Type | V1 | V2 | V3 | V4 |
|--------|-----|-------|---------|------|-----|------|------|------|
| 301 | 14 | c | 1 | t | 293 | 7.8 | 54.3 | 37.9 |
| 302 | 6 | a | 1 | d | 290 | 19.6 | 54.5 | 25.9 |
| 303 | 19 | f | 0 | p | 468 | 12.8 | 57.1 | 30.1 |
| 304 | 17 | b | 1 | p | 462 | 10.0 | 58.2 | 31.8 |
| 305 | 13 | e | 0 | t | 478 | 13.6 | 53.8 | 32.6 |
| 306 | 1 | g | 0 | d | 432 | 17.6 | 58.8 | 23.6 |
| 307 | 3 | c | 1 | d | 444 | 3.8 | 63.5 | 32.7 |
| 308 | 8 | b | 1 | t | 467 | 9.4 | 61.2 | 29.4 |
| 309 | 20 | a | 1 | p | 158 | 10.8 | 60.7 | 28.5 |
| 310 | 15 | e | 0 | p | 433 | 7.6 | 58.7 | 33.7 |
| 311 | 2 | f | 0 | d | 436 | 8.3 | 64.7 | 27.0 |
| 312 | 9 | g | 0 | t | 457 | 6.3 | 60.4 | 33.3 |
| 313 | 16 | g | 0 | p | 459 | 5.7 | 70.1 | 24.2 |
| 314 | 4 | e | 0 | d | 388 | 4.6 | 69.1 | 26.3 |
| 315 | 10 | a | 1 | t | 269 | 9.7 | 56.9 | 33.4 |
| 316 | 5 | b | 1 | d | 405 | 5.9 | 61.5 | 32.6 |
| 317 | 11 | f | 0 | t | 341 | 5.0 | 56.0 | 39.0 |
| 318 | 21 | c | 1 | p | 342 | 3.8 | 50.3 | 45.9 |

V1 = Total Number of Cells Counted

Mean/S.E.M.

| Orbit \ Type | d | p | t |
|--------------|--------------|--------------|--------------|
| 0 | 418.67/51.65 | 453.33/51.65 | 425.33/51.65 |
| 1 | 379.67/51.65 | 320.67/51.65 | 343.00/51.65 |
| | 399.17/36.52 | 387.00/36.52 | 384.17/36.52 |

V2 = % Normocytes

Mean/S.E.M.

| Orbit \ Type | d | p | t |
|--------------|-------------|------------|------------|
| 0 | 10.17/3.066 | 8.70/3.066 | 8.30/3.066 |
| 1 | 9.77/3.066 | 8.20/3.066 | 8.97/3.066 |
| | 9.97/2.168 | 8.45/2.168 | 8.63/2.168 |

V3 = % Echinocytes type I & II

Mean/S.E.M.

| Orbit \ Type | d | p | t |
|--------------|-------------|-------------|-------------|
| 0 | 64.20/2.908 | 61.97/2.908 | 56.73/2.908 |
| 1 | 59.83/2.908 | 56.40/2.908 | 57.47/2.908 |
| | 62.02/2.056 | 59.18/2.056 | 57.10/2.056 |

V4 = % Echinocytes type III

Mean/S.E.M.

| Orbit \ Type | d | p | t | |
|--------------|-------------|-------------|-------------|-------------|
| 0 | 25.63/2.956 | 29.33/2.956 | 34.97/2.956 | 29.98/1.706 |
| 1 | 30.40/2.956 | 35.40/2.956 | 33.57/2.956 | 33.12/1.706 |
| | 28.02/2.090 | 32.37/2.090 | 34.27/2.090 | |

Ausprunk - Transmission Electron Microscopy Data - White Cells

| Sample | Bag | Dewar | Orbit=1 | Type | V1 | V2 | V3 | V4 | V5 |
|--------|-----|-------|---------|------|----|----|----|----|------|
| 341 | 22 | a | 1 | d | 0 | 4 | 3 | 2 | 2.25 |
| 342 | 40 | e | 0 | p | 0 | 2 | 2 | 2 | 1.50 |
| 343 | 24 | f | 0 | d | 2 | 4 | 2 | 2 | 2.50 |
| 344 | 35 | b | 1 | t | 1 | 3 | 3 | 2 | 2.25 |
| 345 | 39 | c | 1 | p | 2 | 2 | 3 | 2 | 2.25 |
| 346 | 31 | g | 0 | t | 1 | 3 | 2 | 1 | 1.75 |
| 347 | 25 | g | 0 | d | 2 | 2 | 1 | 3 | 2.00 |
| 348 | 41 | b | 1 | p | 1 | 3 | 1 | 2 | 1.75 |
| 349 | 32 | e | 0 | t | 1 | 3 | 2 | 2 | 2.00 |
| 350 | 38 | f | 0 | p | 0 | 1 | 1 | 1 | 0.75 |
| 351 | 23 | c | 1 | d | 4 | 1 | 1 | 4 | 2.50 |
| 352 | 29 | a | 1 | t | 3 | 1 | 1 | 3 | 2.00 |
| 353 | 37 | a | 1 | p | 2 | 3 | 1 | 3 | 2.25 |
| 354 | 28 | e | 0 | d | 1 | 3 | 1 | 3 | 2.00 |
| 355 | 36 | g | 0 | p | 2 | 3 | 1 | 2 | 2.00 |
| 356 | 33 | f | 0 | t | 2 | 3 | 2 | 2 | 2.25 |
| 357 | 26 | b | 1 | d | 2 | 3 | 2 | 1 | 2.00 |
| 358 | 34 | c | 1 | t | 2 | 3 | 2 | 3 | 2.50 |

V1 = Degranulated Cells

0 = no cells

1 = occasional cells

2 = half of cells

3 = majority of cells

4 = all cells

Mean/S.E.M.

| Orbit \ Type | d | p | t | |
|--------------|------------|------------|------------|------------|
| 0 | 1.67/0.638 | 0.67/0.638 | 1.33/0.638 | 1.22/0.369 |
| 1 | 2.00/0.638 | 1.67/0.638 | 2.00/0.638 | 1.89/0.369 |
| | 1.83/0.451 | 1.17/0.451 | 1.67/0.451 | |

V2 = Swollen Cytoplasm - same scoring as above

Mean/S.E.M.

| Orbit \ Type | d | p | t | |
|--------------|------------|------------|------------|------------|
| 0 | 3.00/0.577 | 2.00/0.577 | 3.00/0.577 | 2.67/0.333 |
| 1 | 2.67/0.577 | 2.67/0.577 | 2.33/0.577 | 2.56/0.333 |
| | 2.83/0.408 | 2.33/0.408 | 2.67/0.408 | |

v3 = Swollen Nuclear Envelope - same scoring as above

Mean/S.E.M.

| Orbit \ Type | d | p | t | |
|--------------|------------|------------|------------|------------|
| 0 | 1.33/0.471 | 1.33/0.471 | 2.00/0.471 | 1.56/0.272 |
| 1 | 2.00/0.471 | 1.67/0.471 | 2.00/0.471 | 1.89/0.272 |
| | 1.67/0.333 | 1.50/0.333 | 2.00/0.333 | |

v4 = Clumped Chromatin

Mean/S.E.M.

| Orbit \ Type | d | p | t | |
|--------------|------------|------------|------------|------------|
| 0 | 2.67/0.471 | 1.67/0.471 | 1.67/0.471 | 2.00/0.272 |
| 1 | 2.33/0.471 | 2.33/0.471 | 2.67/0.471 | 2.44/0.272 |
| | 2.50/0.333 | 2.00/0.333 | 2.17/0.333 | |

v5 = Average Ranking

Mean/S.E.M.

| Orbit \ Type | d | p | t | |
|--------------|------------|------------|------------|------------|
| 0 | 2.17/0.204 | 1.42/0.204 | 2.00/0.204 | 1.86/0.118 |
| 1 | 2.25/0.204 | 2.08/0.204 | 2.25/0.204 | 2.19/0.118 |
| | 2.21/0.144 | 1.75/0.144 | 2.13/0.144 | |

Ausprunk - Transmission Electron Microscopy Data - Platelets

| Sample | Bag | Dewar | Orbit=1 | Type | V1 | V2 | V3 | V4 | V5 |
|--------|-----|-------|---------|------|----|----|----|----|-----|
| 380 | 45 | h | 0 | d | 0 | 0 | 4 | 4 | 4.0 |
| 381 | 60 | h | 0 | p | 1 | 1 | 4 | 3 | 3.5 |
| 382 | 50 | h | 0 | t | 1 | 1 | 4 | 3 | 3.5 |
| 383 | 48 | d | 1 | d | 1 | 1 | 4 | 3 | 3.5 |
| 384 | 56 | d | 1 | t | - | - | - | - | - |
| 385 | 61 | d | 1 | p | 2 | 0 | 3 | 2 | 2.5 |
| 386 | 62 | d | 1 | p | 1 | 0 | 4 | 3 | 3.5 |
| 387 | 47 | h | 0 | d | 0 | 0 | 4 | 4 | 4.0 |
| 388 | 59 | h | 0 | p | 1 | 0 | 4 | 3 | 3.5 |
| 389 | 43 | d | 1 | d | 0 | 0 | 4 | 4 | 4.0 |
| 390 | 55 | h | 0 | t | 1 | 0 | 4 | 3 | 3.5 |
| 391 | 54 | d | 1 | t | 2 | 0 | 2 | 1 | 1.5 |
| 392 | 63 | h | 0 | p | 1 | 0 | 4 | 3 | 3.5 |
| 393 | 53 | d | 1 | t | 1 | 0 | 4 | 3 | 3.5 |
| 394 | 44 | d | 1 | d | 0 | 0 | 4 | 4 | 4.0 |
| 395 | 46 | h | 0 | d | 0 | 0 | 4 | 4 | 4.0 |
| 396 | 58 | d | 1 | p | 1 | 0 | 4 | 3 | 3.5 |
| 397 | 51 | h | 0 | t | 1 | 0 | 4 | 3 | 3.5 |
| 398 | 98 | h | 0 | t | 1 | 0 | 4 | 3 | 3.5 |
| 399 | 99 | d | 1 | t | 2 | 0 | 4 | 2 | 3.0 |

V1 = Pseudopods

0 = no cells

1 = occasional cells

2 = half of cells

3 = majority of cells

4 = all cells

Mean/S.E.M.

| Orbit \ Type | d | p | t |
|--------------|------------|------------|------------|
| 0 | 0.00/0.226 | 1.00/0.226 | 1.00/0.196 |
| 1 | 0.33/0.226 | 1.33/0.226 | 1.67/0.226 |
| | 0.17/0.160 | 1.17/0.160 | 1.29/0.148 |

orbit p = 0.0400

type p = 0.0003

V2 = Aggregated - same scoring as above

Mean/S.E.M.

| Orbit \ Type | d | p | t |
|--------------|------------|------------|------------|
| 0 | 0.00/0.231 | 0.33/0.231 | 0.25/0.200 |
| | 0.33/0.231 | 0.00/0.231 | 0.00/0.231 |
| | 0.17/0.163 | 0.17/0.163 | 0.14/0.151 |

V3 = Degranulated - same scoring as above

Mean/S.E.M.

| Orbit \ Type | d | p | t | |
|--------------|------------|------------|------------|------------|
| 0 | 4.00/0.292 | 4.00/0.292 | 4.00/0.253 | 4.00/0.160 |
| 1 | 4.00/0.292 | 3.67/0.292 | 3.33/0.292 | 3.67/0.169 |
| | 4.00/0.207 | 3.83/0.207 | 3.71/0.191 | |

V4 = Swollen or Ruptured Cells - same scoring as above

Mean/S.E.M.

| Orbit \ Type | d | p | t | |
|--------------|------------|------------|------------|------------|
| 0 | 4.00/0.292 | 3.00/0.292 | 3.00/0.253 | 3.30/0.160 |
| 1 | 3.67/0.292 | 2.67/0.292 | 2.00/0.292 | 2.78/0.169 |
| | 3.83/0.207 | 2.83/0.207 | 2.57/0.191 | |

orbit p = 0.0428

type p = 0.0014

V5 = Average of V3 and V4

Mean/S.E.M.

| Orbit \ Type | d | p | t | |
|--------------|------------|------------|------------|------------|
| 0 | 4.00/0.277 | 3.50/0.277 | 3.50/0.240 | 3.65/0.152 |
| 1 | 3.83/0.277 | 3.17/0.277 | 2.67/0.277 | 3.22/0.160 |
| | 3.92/0.196 | 3.33/0.196 | 3.14/0.182 | |

type p = 0.0284

Chao 1

| <u>Sample</u> | <u>Bag</u> | <u>Dewar</u> | <u>Orbit=1</u> | <u>Type</u> | <u>VI</u> |
|---------------|------------|--------------|----------------|-------------|-----------|
| 580 | 50 | h | 0 | t | 1.45 |
| 581 | 48 | d | 1 | d | 1.49 |
| 582 | 56 | d | 1 | t | 1.39 |
| 583 | 60 | h | 0 | p | 1.42 |
| 584 | 45 | h | 0 | d | 1.42 |
| 585 | 61 | d | 1 | p | 1.37 |
| 586 | 47 | h | 0 | d | 1.43 |
| 587 | 55 | h | 0 | t | 1.40 |
| 588 | 62 | d | 1 | p | 1.32 |
| 589 | 43 | d | 1 | d | 1.48 |
| 590 | 59 | h | 0 | p | 1.35 |
| 591 | 54 | d | 1 | t | 1.36 |
| 592 | 53 | d | 1 | t | 1.35 |
| 593 | 63 | h | 0 | p | 1.40 |
| 594 | 58 | d | 1 | p | 1.41 |
| 595 | 46 | h | 0 | d | 1.50 |
| 596 | 44 | d | 1 | d | 1.45 |
| 597 | 51 | h | 0 | t | 1.43 |
| 598 | 99 | d | 1 | t | 1.35 |
| 599 | 98 | h | 0 | t | 1.41 |

VI = Platelet Count $\times 10^9$ /ml

Mean/S.E.M.

| Orbit \ Type | d | p | t | |
|--------------|------------|------------|------------|------------|
| 0 | 1.45/0.018 | 1.39/0.018 | 1.42/0.016 | 1.42/0.010 |
| 1 | 1.47/0.018 | 1.37/0.018 | 1.36/0.016 | 1.40/0.010 |
| | 1.46/0.013 | 1.38/0.013 | 1.39/0.011 | |

type p = .0008

Omitting bag number 56:
Mean/S.E.M.

| Orbit \ Type | d | p | t | |
|--------------|------------|------------|------------|------------|
| 0 | 1.45/0.018 | 1.39/0.018 | 1.42/0.016 | 1.42/0.010 |
| 1 | 1.47/0.018 | 1.37/0.018 | 1.35/0.018 | 1.40/0.010 |
| | 1.46/0.013 | 1.38/0.013 | 1.39/0.012 | |

type p = .0011

Chao 2

| <u>Sample</u> | <u>Bag</u> | <u>Dewar</u> | <u>Orbit=1</u> | <u>Type</u> | <u>VI</u> |
|---------------|------------|--------------|----------------|-------------|-----------|
| 580 | 50 | h | 0 | t | 0.000 |
| 581 | 48 | d | 1 | d | 0.000 |
| 582 | 56 | d | 1 | t | 0.088 |
| 583 | 60 | h | 0 | p | 0.000 |
| 584 | 45 | h | 0 | d | 0.000 |
| 585 | 61 | d | 1 | p | 0.000 |
| 586 | 47 | h | 0 | d | 0.000 |
| 587 | 55 | h | 0 | t | 0.000 |
| 588 | 62 | d | 1 | p | 0.000 |
| 589 | 43 | d | 1 | d | 0.000 |
| 590 | 59 | h | 0 | p | 0.000 |
| 591 | 54 | d | 1 | t | 0.059 |
| 592 | 53 | d | 1 | t | 0.000 |
| 593 | 63 | h | 0 | p | 0.000 |
| 594 | 58 | d | 1 | p | 0.000 |
| 595 | 46 | h | 0 | d | 0.000 |
| 596 | 44 | d | 1 | d | 0.000 |
| 597 | 51 | h | 0 | t | 0.000 |
| 598 | 99 | d | 1 | t | 0.007 |
| 599 | 98 | h | 0 | t | 0.000 |

VI = Platelet response to hypotonic Stress (Δ OD / 2 min)

Insufficient data to analyse

Chao 3

| <u>Sample</u> | <u>Bag</u> | <u>Dewar</u> | <u>Orbit=1</u> | <u>Type</u> | <u>V1</u> |
|---------------|------------|--------------|----------------|-------------|-----------|
| 580 | 50 | h | 0 | t | 9.1 |
| 581 | 48 | d | 1 | d | 10.3 |
| 582 | 56 | d | 1 | t | 7.8 |
| 583 | 60 | h | 0 | p | 9.5 |
| 584 | 45 | h | 0 | d | 10.6 |
| 585 | 61 | d | 1 | p | 9.1 |
| 586 | 47 | h | 0 | d | 11.3 |
| 587 | 55 | h | 0 | t | 9.1 |
| 588 | 62 | d | 1 | p | 9.2 |
| 589 | 43 | d | 1 | d | 10.1 |
| 590 | 59 | h | 0 | p | 9.6 |
| 591 | 54 | d | 1 | t | 8.4 |
| 592 | 53 | d | 1 | t | 8.6 |
| 593 | 63 | h | 0 | p | 9.9 |
| 594 | 58 | d | 1 | p | 9.5 |
| 595 | 46 | h | 0 | d | 10.1 |
| 596 | 44 | d | 1 | d | 10.5 |
| 597 | 51 | h | 0 | t | 9.2 |
| 598 | 99 | d | 1 | t | 8.5 |
| 599 | 98 | h | 0 | t | 9.3 |

V1 = Mean Platelet Volume (μm^3)

Mean/S.E.M.

| Orbit \ Type | d | p | t | |
|--------------|-------------|------------|------------|------------|
| 0 | 10.67/0.182 | 9.67/0.182 | 9.18/0.158 | 9.77/0.010 |
| 1 | 10.30/0.182 | 9.27/0.182 | 8.33/0.158 | 9.20/0.010 |
| | 10.48/0.129 | 9.47/0.129 | 8.75/0.112 | |

type p = .0001
orbit p = .0012

Omitting bag number 56:
Mean/S.E.M.

| Orbit \ Type | d | p | t | |
|--------------|-------------|------------|------------|------------|
| 0 | 10.67/0.162 | 9.67/0.162 | 9.18/0.141 | 9.77/0.089 |
| 1 | 10.30/0.162 | 9.27/0.162 | 8.50/0.162 | 9.36/0.094 |
| | 10.48/0.115 | 9.47/0.115 | 8.89/0.106 | |

type p = .0001
orbit p = .0069

Chao 4

| Sample | Bag | Dewar | Orbit=1 | Type | V1 ^② |
|--------|-----|-------|---------|------|-----------------|
| 580 | 50 | h | 0 | t | 6 |
| 581 | 48 | d | 1 | d | 0 |
| 582 | 56 | d | 1 | t | 75 |
| 583 | 60 | h | 0 | p | 0 |
| 584 | 45 | h | 0 | d | 0 |
| 585 | 61 | d | 1 | p | 20 |
| 586 | 47 | h | 0 | d | 24 |
| 587 | 55 | h | 0 | t | 10 |
| 588 | 62 | d | 1 | p | 36 |
| 589 | 43 | d | 1 | d | 21 |
| 590 | 59 | h | 0 | p | 0 |
| 591 | 54 | d | 1 | t | 38 |
| 592 | 53 | d | 1 | t | 20 |
| 593 | 63 | h | 0 | p | 8 |
| 594 | 58 | d | 1 | p | 0 |
| 595 | 46 | h | 0 | d | 0 |
| 596 | 44 | d | 1 | d | 0 |
| 597 | 51 | h | 0 | t | 19 |
| 598 | 99 | d | 1 | t | 21 |
| 599 | 98 | h | 0 | t | 0 |

V1 = Aggregation (by 12.5 µg collagen) % light transmission

② note "trace" interpreted as 0 for purposes of analysis

Mean/S.E.M.

| Orbit \ Type | d | p | t | |
|--------------|------------|-------------|-------------|-------------|
| 0 | 8.00/9.185 | 2.67/9.185 | 8.75/7.954 | 6.70/5.031 |
| 1 | 7.00/9.185 | 18.67/9.185 | 38.50/7.954 | 23.10/5.031 |
| | 7.50/6.495 | 10.67/6.495 | 23.63/5.624 | |

orbit p = .0370

Omitting bag number 56:

Mean/S.E.M.

| Orbit \ Type | d | p | t | |
|--------------|------------|-------------|-------------|-------------|
| 0 | 8.00/6.731 | 2.67/6.731 | 8.75/5.829 | 6.70/3.687 |
| 1 | 7.00/6.731 | 18.67/6.731 | 26.33/6.731 | 17.33/3.886 |
| | 7.50/4.758 | 10.67/4.758 | 16.29/4.406 | |

Shao 5

| Sample | Bag | Dewar | Orbit=1 | Type | VI [@] |
|--------|-----|-------|---------|------|-----------------|
| 580 | 50 | h | 0 | t | 0 |
| 581 | 48 | d | 1 | d | 5 |
| 582 | 56 | d | 1 | t | 44 |
| 583 | 60 | h | 0 | p | 11 |
| 584 | 45 | h | 0 | d | 5 |
| 585 | 61 | d | 1 | p | 0 |
| 586 | 47 | h | 0 | d | 10 |
| 587 | 55 | h | 0 | t | 5 |
| 588 | 62 | d | 1 | p | 11 |
| 589 | 43 | d | 1 | d | 0 |
| 590 | 59 | h | 0 | p | 8 |
| 591 | 54 | d | 1 | t | 45 |
| 592 | 53 | d | 1 | t | 9 |
| 593 | 63 | h | 0 | p | 0 |
| 594 | 58 | d | 1 | p | 0 |
| 595 | 46 | h | 0 | d | 0 |
| 596 | 44 | d | 1 | d | 0 |
| 597 | 51 | h | 0 | t | 0 |
| 598 | 99 | d | 1 | t | 15 |
| 599 | 98 | h | 0 | t | 11 |

1 = Aggregation (by 100 μ M ADP) % light transmission

@ note "trace" interpreted as 0 for purposes of analysis

mean/S.E.M.

| Orbit \ Type | d | p | t | |
|--------------|------------|------------|-------------|-------------|
| 0 | 5.00/5.709 | 6.33/5.709 | 4.00/4.944 | 5.00/3.127 |
| 1 | 1.67/5.709 | 3.67/5.709 | 28.25/4.944 | 12.90/3.127 |
| | 3.33/4.037 | 5.00/4.037 | 16.13/3.496 | |

Submitting bag number 56:

mean/S.E.M.

| Orbit \ Type | d | p | t | |
|--------------|------------|------------|-------------|------------|
| 0 | 5.00/5.159 | 6.33/5.159 | 4.00/4.468 | 5.00/2.826 |
| 1 | 1.67/5.159 | 3.67/5.159 | 23.00/5.159 | 9.44/2.979 |
| | 3.33/3.648 | 5.00/3.648 | 12.14/3.377 | |

Orbit p = 0.0957

Type p = 0.0565

Interaction p = 0.0299

Chao 6

| <u>Sample</u> | <u>Bag</u> | <u>Dewar</u> | <u>Orbit=1</u> | <u>Type</u> | <u>V1</u> |
|---------------|------------|--------------|----------------|-------------|-----------|
| 580 | 50 | h | 0 | t | 0.00 |
| 581 | 48 | d | 1 | d | 0.00 |
| 582 | 56 | d | 1 | t | 2.94 |
| 583 | 60 | h | 0 | p | 0.00 |
| 584 | 45 | h | 0 | d | 0.00 |
| 585 | 61 | d | 1 | p | 0.23 |
| 586 | 47 | h | 0 | d | 0.00 |
| 587 | 55 | h | 0 | t | 0.00 |
| 588 | 62 | d | 1 | p | 0.29 |
| 589 | 43 | d | 1 | d | 0.00 |
| 590 | 59 | h | 0 | p | 0.00 |
| 591 | 54 | d | 1 | t | 1.73 |
| 592 | 53 | d | 1 | t | 0.17 |
| 593 | 63 | h | 0 | p | 0.00 |
| 594 | 58 | d | 1 | p | 0.00 |
| 595 | 46 | h | 0 | d | 0.00 |
| 596 | 44 | d | 1 | d | 0.00 |
| 597 | 51 | h | 0 | t | 0.00 |
| 598 | 99 | d | 1 | t | 0.00 |
| 599 | 98 | h | 0 | t | 0.00 |

V1 = ATP Release (n mol / 10^9 platelets)

Mean/S.E.M.

| Orbit \ Type | d | p | t | |
|--------------|----------|------------|------------|------------|
| 0 | 0 /0.373 | 0 /0.373 | 0 /0.323 | 0 /0.205 |
| 1 | 0 /0.373 | 0.17/0.373 | 1.21/0.323 | 0.54/0.205 |
| | 0 /0.264 | 0.09/0.264 | 0.61/0.229 | |

Omitting bag number 56:

Mean/S.E.M.

| Orbit \ Type | d | p | t | |
|--------------|----------|------------|------------|------------|
| 0 | 0 /0.219 | 0 /0.219 | 0 /0.189 | 0 /0.120 |
| 1 | 0 /0.219 | 0.17/0.219 | 0.63/0.219 | 0.27/0.126 |
| | 0 /0.155 | 0.09/0.155 | 0.27/0.143 | |

Chao 7

| <u>Sample</u> | <u>Bag</u> | <u>Dewar</u> | <u>Orbit=1</u> | <u>Type</u> | | |
|---------------|------------|--------------|----------------|-------------|-------|-------|
| 580 | 50 | h | 0 | t | > | 90 |
| 581 | 48 | d | 1 | d | > | 90 |
| 582 | 56 | d | 1 | t | 30.62 | 29.59 |
| 583 | 60 | h | 0 | p | > | 90 |
| 584 | 45 | h | 0 | d | > | 90 |
| 585 | 61 | d | 1 | p | > | 90 |
| 586 | 47 | h | 0 | d | > | 90 |
| 587 | 55 | h | 0 | t | > | 90 |
| 588 | 62 | d | 1 | p | > | 90 |
| 589 | 43 | d | 1 | d | > | 90 |
| 590 | 59 | h | 0 | p | > | 90 |
| 591 | 54 | d | 1 | t | 43.99 | 43.24 |
| 592 | 53 | d | 1 | t | > | 90 |
| 593 | 63 | h | 0 | p | > | 90 |
| 594 | 58 | d | 1 | p | > | 90 |
| 595 | 46 | h | 0 | d | > | 90 |
| 596 | 44 | d | 1 | d | > | 90 |
| 597 | 51 | h | 0 | t | > | 90 |
| 598 | 99 | d | 1 | t | > | 90 |
| 599 | 98 | h | 0 | t | > | 90 |

V1 = Russell's Viperr Venom Time (sec.)

Insufficient data to analyse

Chao 8

| <u>Sample</u> | <u>Bag</u> | <u>Dewar</u> | <u>Orbit=1</u> | <u>Type</u> | <u>V1</u> |
|---------------|------------|--------------|----------------|-------------|-----------|
| 580 | 50 | h | 0 | t | 4.7 |
| 581 | 48 | d | 1 | d | 4.5 |
| 582 | 56 | d | 1 | t | 57.3 |
| 583 | 60 | h | 0 | p | 0.0 |
| 584 | 45 | h | 0 | d | 0.0 |
| 585 | 61 | d | 1 | p | 1.9 |
| 586 | 47 | h | 0 | d | 0.0 |
| 587 | 55 | h | 0 | t | 5.0 |
| 588 | 62 | d | 1 | p | 0.0 |
| 589 | 43 | d | 1 | d | 6.1 |
| 590 | 59 | h | 0 | p | 19.2 |
| 591 | 54 | d | 1 | t | 37.9 |
| 592 | 53 | d | 1 | t | 0.0 |
| 593 | 63 | h | 0 | p | 0.0 |
| 594 | 58 | d | 1 | p | 1.9 |
| 595 | 46 | h | 0 | d | 2.1 |
| 596 | 44 | d | 1 | d | 3.2 |
| 597 | 51 | h | 0 | t | 0.0 |
| 598 | 99 | d | 1 | t | 11.3 |
| 599 | 98 | h | 0 | t | 0.0 |

V1 = Serotonin Uptake % (10 min. incubation)

Mean/S.E.M.

| Orbit \ Type | d | p | t | |
|--------------|------------|------------|-------------|-------------|
| 0 | 0.70/7.385 | 6.40/7.385 | 2.43/6.396 | 3.10/4.045 |
| 1 | 4.60/7.385 | 1.27/7.385 | 26.63/6.396 | 12.41/4.045 |
| | 2.65/5.222 | 3.83/5.222 | 14.53/4.522 | |

Omitting bag number 56:

Mean/S.E.M.

| Orbit \ Type | d | p | t | |
|--------------|------------|------------|-------------|------------|
| 0 | 0.70/5.154 | 6.40/5.154 | 2.43/4.464 | 3.10/2.823 |
| 1 | 4.60/5.154 | 1.27/5.154 | 16.40/5.154 | 7.42/2.976 |
| | 2.65/3.645 | 3.83/3.645 | 8.41/3.374 | |

Chao 9

| <u>Sample</u> | <u>Bag</u> | <u>Dewar</u> | <u>Orbit=1</u> | <u>Type</u> | <u>V1</u> |
|---------------|------------|--------------|----------------|-------------|-----------|
| 580 | 50 | h | 0 | t | 0.0 |
| 581 | 48 | d | 1 | d | 0.0 |
| 582 | 56 | d | 1 | t | 35.9 |
| 583 | 60 | h | 0 | p | 0.0 |
| 584 | 45 | h | 0 | d | 0.0 |
| 585 | 61 | d | 1 | p | 0.0 |
| 586 | 47 | h | 0 | d | 0.0 |
| 587 | 55 | h | 0 | t | 0.0 |
| 588 | 62 | d | 1 | p | 0.0 |
| 589 | 43 | d | 1 | d | 0.0 |
| 590 | 59 | h | 0 | p | 0.0 |
| 591 | 54 | d | 1 | t | 29.8 |
| 592 | 53 | d | 1 | t | 0.0 |
| 593 | 63 | h | 0 | p | 0.0 |
| 594 | 58 | d | 1 | p | 0.0 |
| 595 | 46 | h | 0 | d | 0.0 |
| 596 | 44 | d | 1 | d | 0.0 |
| 597 | 51 | h | 0 | t | 0.0 |
| 598 | 99 | d | 1 | t | 0.0 |
| 599 | 98 | h | 0 | t | 0.0 |

V1 = Serotonin Release % (4 min. after stimulation)

Insufficient data to analyse.

Chao 10

| <u>Sample</u> | <u>Bag</u> | <u>Dewar</u> | <u>Orbit=1</u> | <u>Type</u> | <u>V1</u> |
|---------------|------------|--------------|----------------|-------------|-----------|
| 580 | 50 | h | 0 | t | 62.32 |
| 581 | 48 | d | 1 | d | 53.93 |
| 582 | 56 | d | 1 | t | 36.96 |
| 583 | 60 | h | 0 | p | 57.87 |
| 584 | 45 | h | 0 | d | 65.31 |
| 585 | 61 | d | 1 | p | 40.78 |
| 586 | 47 | h | 0 | d | 74.87 |
| 587 | 55 | h | 0 | t | 59.51 |
| 588 | 62 | d | 1 | p | 42.98 |
| 589 | 43 | d | 1 | d | 55.07 |
| 590 | 59 | h | 0 | p | 43.62 |
| 591 | 54 | d | 1 | t | 35.04 |
| 592 | 53 | d | 1 | t | 49.27 |
| 593 | 63 | h | 0 | p | 52.70 |
| 594 | 58 | d | 1 | p | 50.46 |
| 595 | 46 | h | 0 | d | 48.58 |
| 596 | 44 | d | 1 | d | 82.80 |
| 597 | 51 | h | 0 | t | 42.65 |
| 598 | 99 | d | 1 | t | 48.28 |
| 599 | 98 | h | 0 | t | 49.32 |

V1 = β - Thromboglobulin ($\mu\text{g} / \text{ml}$)

Mean/S.E.M.

| Orbit \ Type | d | p | t | |
|--------------|-------------|-------------|-------------|-------------|
| 0 | 62.92/5.894 | 51.40/5.894 | 53.45/5.104 | 55.68/3.228 |
| 1 | 63.93/5.894 | 44.74/5.894 | 42.39/5.104 | 49.56/3.228 |
| | 63.43/4.168 | 48.07/4.168 | 47.92/3.609 | |

type p = .0257

Omitting bag number 56:

Mean/S.E.M.

| Orbit \ Type | d | p | t | |
|--------------|-------------|-------------|-------------|-------------|
| 0 | 62.92/6.033 | 51.40/6.033 | 53.45/5.225 | 55.68/3.305 |
| 1 | 63.93/6.033 | 44.74/6.033 | 44.20/6.033 | 50.96/3.483 |
| | 63.43/4.266 | 48.07/4.266 | 49.48/3.950 | |

type p = .0434

Chao 11

| Sample | Bag | Dewar | Orbit=1 | Type | VI [@] |
|--------|-----|-------|---------|------|-----------------|
| 580 | 50 | h | 0 | t | 5.2 |
| 581 | 48 | d | 1 | d | 4.0 |
| 582 | 56 | d | 1 | t | 1.3 |
| 583 | 60 | h | 0 | p | 7.4 |
| 584 | 45 | h | 0 | d | 16.8 |
| 585 | 61 | d | 1 | p | 25.0 |
| 586 | 47 | h | 0 | d | 12.0 |
| 587 | 55 | h | 0 | t | 5.3 |
| 588 | 62 | d | 1 | p | 1.5 |
| 589 | 43 | d | 1 | d | 2.0 |
| 590 | 59 | h | 0 | p | 5.2 |
| 591 | 54 | d | 1 | t | 1.6 |
| 592 | 53 | d | 1 | t | 1.1 |
| 593 | 63 | h | 0 | p | 8.0 |
| 594 | 58 | d | 1 | p | 1.8 |
| 595 | 46 | h | 0 | d | 20.0 |
| 596 | 44 | d | 1 | d | 25.0 |
| 597 | 51 | h | 0 | t | 20.0 |
| 598 | 99 | d | 1 | t | 1.4 |
| 599 | 98 | h | 0 | t | 6.8 |

VI[@] = Thromboxane B₂

Note "> 20" interpreted as 25 for purposes of analysis

Mean/S.E.M.

| Orbit \ Type | d | p | t | |
|--------------|-------------|------------|------------|-------------|
| 0 | 16.27/4.574 | 6.87/4.574 | 9.33/3.961 | 10.67/2.505 |
| 1 | 10.33/4.574 | 9.43/4.574 | 1.35/3.961 | 6.47/2.505 |
| | 13.30/3.234 | 8.15/3.234 | 5.34/2.801 | |

Omitting bag number 56:

Mean/S.E.M.

| Orbit \ Type | d | p | t | |
|--------------|-------------|------------|------------|-------------|
| 0 | 16.27/4.746 | 6.87/4.746 | 9.33/4.111 | 10.67/2.600 |
| 1 | 10.33/4.746 | 9.43/4.746 | 1.37/4.746 | 7.04/2.740 |
| | 13.30/3.356 | 8.15/3.356 | 5.91/3.107 | |

Chao 12

| <u>Sample</u> | <u>Bag</u> | <u>Dewar</u> | <u>Orbit=1</u> | <u>Type</u> | <u>V1</u> |
|---------------|------------|--------------|----------------|-------------|-----------|
| 580 | 50 | h | 0 | t | 22.1 |
| 581 | 48 | d | 1 | d | 22.2 |
| 582 | 56 | d | 1 | t | 16.7 |
| 583 | 60 | h | 0 | p | 21.7 |
| 584 | 45 | h | 0 | d | 23.9 |
| 585 | 61 | d | 1 | p | 21.3 |
| 586 | 47 | h | 0 | d | 24.0 |
| 587 | 55 | h | 0 | t | 23.1 |
| 588 | 62 | d | 1 | p | 23.4 |
| 589 | 43 | d | 1 | d | 24.4 |
| 590 | 59 | h | 0 | p | 23.3 |
| 591 | 54 | d | 1 | t | 19.0 |
| 592 | 53 | d | 1 | t | 20.8 |
| 593 | 63 | h | 0 | p | 24.0 |
| 594 | 58 | d | 1 | p | 23.7 |
| 595 | 46 | h | 0 | d | 24.8 |
| 596 | 44 | d | 1 | d | 22.3 |
| 597 | 51 | h | 0 | t | 24.7 |
| 598 | 99 | d | 1 | t | 23.2 |
| 599 | 98 | h | 0 | t | 24.4 |

V1 = Lactate (mM)

Mean/S.E.M.

| Orbit \ Type | d | p | t | |
|--------------|-------------|-------------|-------------|-------------|
| 0 | 24.23/0.937 | 23.00/0.937 | 23.58/0.811 | 23.60/0.513 |
| 1 | 22.97/0.937 | 22.80/0.937 | 19.93/0.811 | 21.70/0.513 |
| | 23.60/0.663 | 22.90/0.663 | 21.75/0.574 | |

orbit p = 0.0203

Omitting bag number 56:
Mean/S.E.M.

| Orbit \ Type | d | p | t | |
|--------------|-------------|-------------|-------------|-------------|
| 0 | 24.23/0.768 | 23.00/0.768 | 23.58/0.665 | 23.60/0.421 |
| 1 | 22.97/0.768 | 22.80/0.768 | 21.00/0.768 | 22.26/0.443 |
| | 23.60/0.543 | 22.90/0.543 | 22.47/0.503 | |

orbit p = 0.0465

Chao 13

| <u>Sample</u> | <u>Bag</u> | <u>Dewar</u> | <u>Orbit=1</u> | <u>Type</u> | <u>V1</u> |
|---------------|------------|--------------|----------------|-------------|-----------|
| 580 | 50 | h | 0 | t | 38 |
| 581 | 48 | d | 1 | d | 23 |
| 582 | 56 | d | 1 | t | 141 |
| 583 | 60 | h | 0 | p | 47 |
| 584 | 45 | h | 0 | d | 33 |
| 585 | 61 | d | 1 | p | 24 |
| 586 | 47 | h | 0 | d | 38 |
| 587 | 55 | h | 0 | t | 22 |
| 588 | 62 | d | 1 | p | 26 |
| 589 | 43 | d | 1 | d | 20 |
| 590 | 59 | h | 0 | p | 28 |
| 591 | 54 | d | 1 | t | 86 |
| 592 | 53 | d | 1 | t | 22 |
| 593 | 63 | h | 0 | p | 23 |
| 594 | 58 | d | 1 | p | 14 |
| 595 | 46 | h | 0 | d | 25 |
| 596 | 44 | d | 1 | d | 21 |
| 597 | 51 | h | 0 | t | 20 |
| 598 | 99 | d | 1 | t | 18 |
| 599 | 98 | h | 0 | t | 25 |

V1 = Glucose (mg / 100 ml)

Mean/S.E.M.

| Orbit \ Type | d | p | t | |
|--------------|--------------|--------------|--------------|-------------|
| 0 | 32.00/16.149 | 32.67/16.149 | 26.25/13.986 | 29.90/8.845 |
| 1 | 21.33/16.149 | 21.33/16.149 | 66.75/13.986 | 39.50/8.845 |
| | 26.67/11.419 | 27.00/11.419 | 46.50/9.889 | |

Omitting bag number 56:

Mean/S.E.M.

| Orbit \ Type | d | p | t | |
|--------------|-------------|-------------|-------------|-------------|
| 0 | 32.00/9.611 | 32.67/9.611 | 25.25/8.324 | 29.90/5.264 |
| 1 | 21.33/9.611 | 21.33/9.611 | 42.00/9.611 | 28.22/5.549 |
| | 26.67/6.796 | 27.00/6.796 | 33.00/6.292 | |

Curby 1 - Red Cells Stress Test

| Sample | Bag | Dewar | Orbit=1 | Type | V1 | V2 | V3 |
|--------|-----|-------|---------|------|---------|---------|----|
| 401 | 17 | b | 1 | p | 27.400 | 14.810 | 22 |
| 402 | 1 | g | 0 | d | 133.000 | 58.620 | 23 |
| 403 | 13 | e | 0 | t | 332.200 | 204.700 | 23 |
| 404 | 19 | f | 0 | p | 248.200 | 123.100 | 16 |
| 405 | 14 | c | 1 | t | 184.600 | 104.000 | 22 |
| 406 | 6 | a | 1 | d | 0.000 | 0.000 | 18 |
| 407 | 8 | b | 1 | t | 149.600 | 91.770 | 24 |
| 408 | 2 | f | 0 | d | 140.400 | 92.630 | 30 |
| 409 | 20 | a | 1 | p | 75.600 | 42.100 | 21 |
| 410 | 9 | g | 0 | t | 148.000 | 108.600 | 22 |
| 411 | 3 | c | 1 | d | 268.600 | 173.300 | 23 |
| 412 | 15 | e | 0 | p | 0.000 | 0.000 | 28 |
| 413 | 11 | f | 0 | t | 112.800 | 60.390 | 23 |
| 414 | 4 | e | 0 | d | 8.199 | 5.827 | 23 |
| 415 | 10 | a | 1 | t | 141.200 | 90.080 | 12 |
| 416 | 16 | g | 0 | p | 256.000 | 26.070 | 20 |
| 417 | 21 | c | 1 | p | 163.200 | 58.180 | 23 |
| 418 | 5 | b | 1 | d | 65.200 | 45.020 | 27 |

V1 = Loss at 20 min., count/ml $\times 10^6$

Mean/S.E.M.

| Orbit \ Type | d | p | t | |
|--------------|---------------|---------------|---------------|---------------|
| 0 | 93.87/60.323 | 168.07/60.323 | 197.67/60.323 | 153.20/34.828 |
| 1 | 111.27/60.323 | 88.73/60.323 | 158.47/60.323 | 119.49/34.828 |
| | 102.57/42.655 | 128.40/42.655 | 178.07/42.655 | |

V2 = Oversized Particle Loss at 20 min., count/ml $\times 10^4$

Mean/S.E.M.

| Orbit \ Type | d | p | t | |
|--------------|--------------|--------------|---------------|--------------|
| 0 | 52.36/33.452 | 49.72/33.452 | 124.56/33.452 | 75.55/19.314 |
| 1 | 72.77/33.452 | 38.36/33.452 | 95.28/33.452 | 68.81/19.314 |
| | 62.57/23.654 | 44.04/23.654 | 109.92/23.654 | |

V3 = Peak Channel Shift at 20 min.

Mean/S.E.M.

| Orbit \ Type | d | p | t | |
|--------------|-------------|-------------|-------------|-------------|
| 0 | 25.33/2.546 | 21.33/2.546 | 22.67/2.546 | 23.11/1.470 |
| 1 | 22.67/2.546 | 22.00/2.546 | 19.33/2.546 | 21.33/1.470 |
| | 24.00/1.800 | 21.67/1.800 | 21.00/1.800 | |

Curby 2 - White Cell Distribution

| Sample | Bag | Dewar | Orbit=1 | Type | V1 | V2 | V3 | V4 |
|--------|-----|-------|---------|------|-------|--------|-----|-------|
| 441 | 24 | f | 0 | d | 3.688 | 9.588 | 90 | 0.225 |
| 442 | 35 | b | 1 | t | 3.802 | 10.670 | 94 | 1.010 |
| 443 | 31 | g | 0 | t | 2.312 | 10.100 | 95 | 1.041 |
| 444 | 22 | a | 1 | d | 2.558 | 9.372 | 96 | 0.936 |
| 445 | 40 | e | 0 | p | 5.807 | 18.630 | 110 | 0.635 |
| 446 | 39 | c | 1 | p | 3.964 | 11.080 | 100 | 0.712 |
| 447 | 29 | a | 1 | t | 6.086 | 15.600 | 105 | 1.064 |
| 448 | 25 | g | 0 | d | 4.080 | 10.500 | 89 | 0.991 |
| 449 | 32 | e | 0 | t | 7.678 | 20.800 | 104 | 0.779 |
| 450 | 38 | f | 0 | p | 3.263 | 9.108 | 98 | 0.514 |
| 451 | 41 | b | 1 | p | 2.877 | 7.132 | 90 | 0.372 |
| 452 | 23 | c | 1 | d | 2.624 | 9.736 | 87 | 0.806 |
| 453 | 33 | f | 0 | t | 4.606 | 11.500 | 95 | 0.724 |
| 454 | 36 | g | 0 | p | 3.421 | 10.240 | 94 | 1.040 |
| 455 | 28 | e | 0 | d | 7.360 | 20.440 | 89 | 0.785 |
| 456 | 26 | b | 1 | d | 2.976 | 10.350 | 89 | 1.107 |
| 457 | 34 | c | 1 | t | 4.126 | 10.070 | 90 | 1.060 |
| 458 | 37 | a | 1 | p | 6.831 | 22.390 | 99 | 0.726 |

V1 = 1 - 254 count/ml $\times 10^7$

Mean/S.E.M.

| Orbit \ Type | d | p | t |
|--------------|------------|------------|------------|
| 0 | 5.04/1.030 | 4.16/1.030 | 4.87/1.030 |
| 1 | 2.72/1.030 | 4.56/1.030 | 4.67/1.030 |
| | 3.88/0.728 | 4.36/0.728 | 4.77/0.728 |

V2 = > 254 , count/ml $\times 10^6$

Mean/S.E.M.

| Orbit \ Type | d | p | t |
|--------------|-------------|-------------|-------------|
| 0 | 13.51/3.067 | 12.66/3.067 | 14.13/3.067 |
| 1 | 9.82/3.067 | 13.53/3.067 | 12.11/3.067 |
| | 11.66/2.168 | 13.10/2.168 | 13.12/2.168 |

V3 = Peak Channel

Mean/S.E.M.

| Orbit \ Type | d | p | t |
|--------------|-------------|--------------|-------------|
| 0 | 89.33/3.413 | 100.67/3.413 | 98.00/3.413 |
| 1 | 90.67/3.413 | 96.33/3.413 | 96.33/3.413 |
| | 90.00/2.413 | 98.50/2.413 | 97.17/2.413 |

V4 = Delay Factor - not analysed

Curby 3 - Platelets Cell Distribution

| Sample | Bag | Dewar | Orbit=1 | Type | V1 | V2 | V3 | V4 |
|--------|-----|-------|---------|------|-------|------|----|-------|
| 481 | 50 | h | 0 | t | 1.249 | 3.4 | 12 | 5.215 |
| 482 | 48 | d | 1 | d | 1.632 | 4.6 | 12 | 7.512 |
| 483 | 61 | d | 1 | p | 1.465 | 6.4 | 12 | 6.978 |
| 484 | 60 | h | 0 | p | 1.027 | 5.6 | 14 | 7.764 |
| 485 | 45 | h | 0 | d | 1.306 | 6.0 | 14 | 7.055 |
| 486 | 43 | d | 1 | d | 1.699 | 6.8 | 15 | 7.372 |
| 487 | 55 | h | 0 | t | 2.258 | 6.4 | 15 | 1.475 |
| 488 | 47 | h | 0 | d | 2.406 | 3.4 | 23 | 1.541 |
| 489 | 62 | d | 1 | p | 1.161 | 2.8 | 13 | 6.295 |
| 490 | 59 | h | 0 | p | 1.645 | 7.8 | 14 | 6.338 |
| 491 | 54 | d | 1 | t | 1.496 | 6.8 | 14 | 6.663 |
| 492 | 58 | d | 1 | p | 1.411 | 6.2 | 14 | 2.588 |
| 493 | 51 | h | 0 | t | 1.428 | 10.2 | 13 | 6.590 |
| 494 | 46 | h | 0 | d | 1.663 | 7.2 | 14 | 6.700 |
| 495 | 53 | d | 1 | t | 1.279 | 5.2 | 14 | 7.435 |
| 496 | 44 | d | 1 | d | 1.483 | 7.8 | 12 | 6.211 |
| 497 | 63 | h | 0 | p | 1.280 | 5.6 | 12 | 6.997 |
| 498 | 98 | h | 0 | t | 1.654 | 6.8 | 12 | 6.691 |
| 499 | 99 | d | 1 | t | 1.524 | 9.2 | 12 | 5.813 |

V1 = 1 - 254 count/ml $\times 10^9$
Mean/S.E.M.

| Orbit \ Type | d | p | t | |
|--------------|------------|------------|------------|------------|
| 0 | 1.79/0.197 | 1.32/0.197 | 1.65/0.171 | 1.59/0.108 |
| 1 | 1.60/0.197 | 1.35/0.197 | 1.43/0.197 | 1.46/0.144 |
| | 1.70/0.139 | 1.33/0.139 | 1.56/0.129 | |

V2 = > 254 , count/ml $\times 10^6$
Mean/S.E.M.

| Orbit \ Type | d | p | t | |
|--------------|------------|------------|------------|------------|
| 0 | 5.53/1.195 | 6.33/1.195 | 6.70/1.035 | 6.24/0.654 |
| 1 | 6.40/1.195 | 5.13/1.195 | 7.07/1.195 | 6.20/0.690 |
| | 5.97/0.845 | 5.73/0.845 | 6.86/0.782 | |

V3 = Peak Channel
Mean/S.E.M.

| Orbit \ Type | d | p | t | |
|--------------|-------------|-------------|-------------|-------------|
| 0 | 17.00/1.371 | 13.33/1.371 | 13.00/1.188 | 14.30/0.751 |
| 1 | 13.00/1.371 | 13.00/1.371 | 13.33/1.371 | 13.11/0.792 |
| | 15.00/0.970 | 13.17/0.970 | 13.14/0.898 | |

V4 = Delay Factor - not analysed

Curby 4 - Red Cells Cell Distribution

| Sample | Bag | Dewar | Orbit=1 | Type | V1 | V2 | V3 | V4 |
|--------|-----|-------|---------|------|-------|-------|-----|-------|
| 401 | 17 | b | 1 | p | 3.388 | 7.84 | 110 | 1.564 |
| 402 | 1 | g | 0 | d | 3.871 | 14.02 | 108 | 0.491 |
| 403 | 13 | e | 0 | t | 3.446 | 12.66 | 106 | 1.315 |
| 404 | 19 | f | 0 | p | 4.324 | 67.62 | 140 | 1.735 |
| 405 | 14 | c | 1 | t | 3.435 | 14.26 | 106 | 1.331 |
| 406 | 6 | a | 1 | d | 3.898 | 13.46 | 106 | 1.271 |
| 407 | 8 | b | 1 | t | 3.534 | 14.56 | 110 | 1.257 |
| 408 | 2 | f | 0 | d | 3.109 | 21.06 | 112 | 1.633 |
| 409 | 20 | a | 1 | p | 3.249 | 10.76 | 105 | 1.377 |
| 410 | 9 | g | 0 | t | 3.295 | 16.88 | 110 | 1.357 |
| 411 | 3 | c | 1 | d | 3.737 | 21.64 | 112 | 1.552 |
| 412 | 15 | e | 0 | p | 3.906 | 18.58 | 111 | 1.346 |
| 413 | 11 | f | 0 | t | 3.468 | 12.90 | 108 | 1.321 |
| 414 | 4 | e | 0 | d | 3.361 | 8.74 | 108 | 1.374 |
| 415 | 10 | a | 1 | t | 3.421 | 10.28 | 100 | 1.339 |
| 416 | 16 | g | 0 | p | 3.552 | 10.52 | 106 | 1.321 |
| 417 | 21 | c | 1 | p | 3.839 | 10.80 | 108 | 1.539 |
| 418 | 5 | b | 1 | d | 3.152 | 4.58 | 100 | 1.642 |

V1 = 1 - 254 count/ml $\times 10^9$

Mean/S.E.M.

| Orbit \ Type | d | p | t |
|--------------|------------|------------|------------|
| 0 | 3.45/0.177 | 3.93/0.177 | 3.40/0.177 |
| 1 | 3.60/0.177 | 3.49/0.177 | 3.46/0.177 |
| | 3.52/0.125 | 3.71/0.125 | 3.43/0.125 |

V2 = > 254 , count/ml $\times 10^7$

Mean/S.E.M.

| Orbit \ Type | d | p | t |
|--------------|-------------|-------------|-------------|
| 0 | 14.61/7.747 | 32.24/7.747 | 14.15/7.747 |
| 1 | 13.22/7.747 | 9.80/7.747 | 13.03/7.747 |
| | 13.92/5.478 | 21.02/5.478 | 13.59/5.478 |

V3 = Peak Channel

Mean/S.E.M.

| Orbit \ Type | d | p | t |
|--------------|--------------|--------------|--------------|
| 0 | 109.33/0.177 | 119.00/0.177 | 108.00/0.177 |
| 1 | 106.00/0.177 | 107.67/0.177 | 105.33/0.177 |
| | 107.67/0.125 | 113.33/0.125 | 106.67/0.125 |

V4 = Delay Factor - not analysed

Jacobson 1 - White Blood Cells

| Sample | Bag | Dewar | Orbit=1 | Type | V1 | V2 | V3 |
|--------|-----|-------|---------|------|-------|------|-------|
| 871 | 22 | a | 1 | d | 6.945 | 42.2 | 228.1 |
| 872 | 35 | b | 1 | t | 7.073 | 25.5 | 263.7 |
| 873 | 31 | g | 0 | t | 7.092 | 23.9 | 259.4 |
| 874 | 24 | f | 0 | d | 6.965 | 38.7 | 220.5 |
| 875 | 39 | c | 1 | p | 7.051 | 27.9 | 259.1 |
| 876 | 40 | e | 0 | p | 7.093 | 24.9 | 255.3 |
| 877 | 25 | g | 0 | d | 6.931 | 41.5 | 220.5 |
| 878 | 32 | e | 0 | t | 7.101 | 22.0 | 263.4 |
| 879 | 29 | a | 1 | t | 7.087 | 24.5 | 252.6 |
| 880 | 38 | f | 0 | p | 7.052 | 24.5 | 269.9 |
| 881 | 41 | b | 1 | p | 7.095 | 24.6 | 236.5 |
| 882 | 23 | c | 1 | d | 6.893 | 44.5 | 226.6 |
| 883 | 37 | a | 1 | p | 7.031 | 28.8 | 261.5 |
| 884 | 28 | e | 0 | d | 6.907 | 42.2 | 225.5 |
| 885 | 34 | c | 1 | t | 7.079 | 25.5 | 259.0 |
| 886 | 33 | f | 0 | t | 7.067 | 23.8 | 248.0 |
| 887 | 36 | g | 0 | p | 7.039 | 26.5 | 253.2 |
| 888 | 26 | b | 1 | d | 6.930 | 42.4 | 240.7 |

V1 = pH

Mean/S.E.M.

| Orbit \ Type | d | p | t | |
|--------------|------------|------------|------------|------------|
| 0 | 6.93/0.015 | 7.06/0.015 | 7.09/0.015 | 7.03/0.008 |
| 1 | 6.92/0.015 | 7.06/0.015 | 7.08/0.015 | 7.02/0.008 |
| | 6.93/0.010 | 7.06/0.010 | 7.08/0.010 | |

type p = 0.0001

V2 = PCO₂ - mm Hg

Mean/S.E.M.

| Orbit \ Type | d | p | t | |
|--------------|-------------|-------------|-------------|-------------|
| 0 | 40.00/0.835 | 25.30/0.835 | 23.23/0.835 | 29.77/0.482 |
| 1 | 43.03/0.835 | 27.10/0.835 | 25.17/0.835 | 31.77/0.482 |
| | 41.92/0.590 | 26.20/0.590 | 24.20/0.590 | |

type p = 0.0001

orbit p = 0.0129

V3 = PO₂ - mm Hg

Mean/S.E.M.

| Orbit \ Type | d | p | t | |
|--------------|--------------|--------------|--------------|--------------|
| 0 | 222.17/4.923 | 259.47/4.923 | 256.93/4.923 | 246.19/2.842 |
| 1 | 231.00/4.923 | 252.37/4.923 | 258.43/4.923 | 247.53/2.842 |
| | 226.98/3.481 | 255.92/3.481 | 257.68/3.481 | |

type p = 0.0001

Jacobson 2 - Red Blood Cells

| Sample | Bag | Dewar | Orbit=1 | Type | V1 | V2 | V3 |
|--------|-----|-------|---------|------|-------|-------|------|
| 161 | 13 | e | 0 | t | 6.763 | 122.1 | 52.4 |
| 162 | 17 | b | 1 | p | 6.707 | 134.5 | 49.9 |
| 163 | 19 | f | 0 | p | 6.743 | 133.8 | 50.1 |
| 164 | 1 | g | 0 | d | 6.711 | 155.2 | 37.0 |
| 165 | 6 | a | 1 | d | 6.738 | 149.4 | 36.6 |
| 166 | 14 | c | 1 | t | 6.730 | 133.9 | 48.2 |
| 167 | 3 | c | 1 | d | 6.672 | 169.8 | 34.1 |
| 168 | 9 | g | 0 | t | 6.747 | 128.2 | 48.8 |
| 169 | 8 | b | 1 | t | 6.766 | 129.0 | 49.8 |
| 170 | 2 | f | 0 | d | 6.710 | 157.5 | 36.4 |
| 171 | 15 | e | 0 | p | 6.740 | 134.3 | 45.8 |
| 172 | 20 | a | 1 | p | 6.712 | 143.4 | 51.0 |
| 173 | 4 | e | 0 | d | 6.744 | 145.8 | 35.7 |
| 174 | 16 | g | 0 | p | 6.758 | 132.1 | 51.5 |
| 175 | 5 | b | 1 | d | 6.695 | 161.2 | 35.9 |
| 176 | 11 | f | 0 | t | 6.767 | 126.0 | 47.8 |
| 177 | 21 | c | 1 | p | 6.728 | 143.5 | 46.2 |
| 178 | 10 | a | 1 | t | 6.742 | 134.1 | 47.6 |

V1 = pH

Mean/S.E.M.

| Orbit \ Type | d | p | t | |
|--------------|------------|------------|------------|------------|
| 0 | 6.72/0.011 | 6.75/0.011 | 6.76/0.011 | 6.74/0.006 |
| 1 | 6.70/9.611 | 6.72/9.611 | 6.75/9.611 | 6.72/0.006 |
| | 6.71/0.008 | 6.73/0.008 | 6.75/0.008 | |

type p = 0.0099

orbit p = 0.0336

V2 = PCO₂ - mm Hg

Mean/S.E.M.

| Orbit \ Type | d | p | t | |
|--------------|--------------|--------------|--------------|--------------|
| 0 | 152.83/3.243 | 133.40/3.243 | 125.43/3.243 | 137.22/1.872 |
| 1 | 160.13/3.243 | 140.47/3.243 | 132.33/3.243 | 144.31/1.872 |
| | 156.48/2.293 | 136.93/2.293 | 128.88/2.293 | |

type p = 0.0001

orbit p = 0.0201

V3 = PO₂ - mm Hg

Mean/S.E.M.

| Orbit \ Type | d | p | t | |
|--------------|-------------|-------------|-------------|-------------|
| 0 | 36.37/1.164 | 49.13/1.164 | 49.67/1.164 | 45.06/0.672 |
| 1 | 35.53/1.164 | 49.03/1.164 | 48.53/1.164 | 44.37/0.672 |
| | 35.95/0.823 | 49.08/0.823 | 49.10/0.823 | |

type p = 0.0001

Jacobson 3 - Platelets

| Sample | Bag | Dewar | Orbit=1 | Type | V1 | V2 | V3 |
|--------|-----|-------|---------|------|-------|------|-------|
| 140 | 61 | d | 1 | p | 5.811 | 20.3 | 170.7 |
| 141 | 48 | d | 1 | d | 5.663 | 10.8 | 185.2 |
| 142 | 50 | h | 0 | t | 5.833 | 7.3 | 221.9 |
| 143 | 56 | d | 1 | t | 6.544 | 22.6 | 186.9 |
| 144 | 60 | h | 0 | p | 5.880 | 11.5 | 202.1 |
| 145 | 45 | h | 0 | d | 5.712 | 9.2 | 195.3 |
| 146 | 54 | d | 1 | t | 6.237 | 29.9 | 145.5 |
| 147 | 55 | h | 0 | t | 5.748 | 7.2 | 201.4 |
| 148 | 43 | d | 1 | d | 5.659 | 8.7 | 194.2 |
| 149 | 47 | h | 0 | d | 5.734 | 8.9 | 207.6 |
| 150 | 62 | d | 1 | p | 5.814 | 18.3 | 189.1 |
| 151 | 59 | h | 0 | p | 5.826 | 8.0 | 214.2 |
| 152 | 51 | h | 0 | t | 5.732 | 6.8 | 211.0 |
| 153 | 63 | h | 0 | p | 5.722 | 6.4 | 206.8 |
| 154 | 58 | d | 1 | p | 5.729 | 10.6 | 203.4 |
| 155 | 44 | d | 1 | d | 5.662 | 10.8 | 184.8 |
| 156 | 53 | d | 1 | t | 5.781 | 12.1 | 191.6 |
| 157 | 46 | h | 0 | d | 5.680 | 8.6 | 203.5 |
| 158 | 99 | d | 1 | t | 5.763 | 12.5 | 189.7 |
| 159 | 98 | h | 0 | t | 5.753 | 6.5 | 205.0 |

V1 = pH

Mean/S.E.M.

| Orbit \ Type | d | p | t |
|--------------|------------|------------|------------|
| 0 | 5.71/0.104 | 5.81/0.104 | 5.77/0.090 |
| 1 | 5.66/0.104 | 5.78/0.104 | 5.87/0.057 |
| | 5.69/0.074 | 5.80/0.074 | 5.92/0.064 |

omitting bag number 56:

Mean/S.E.M.

| Orbit \ Type | d | p | t |
|--------------|------------|------------|------------|
| 0 | 5.71/0.066 | 5.81/0.066 | 5.77/0.057 |
| 1 | 5.66/0.066 | 5.78/0.066 | 5.93/0.066 |
| | 5.69/0.047 | 5.80/0.047 | 5.84/0.043 |

V2 = PCO₂ - mm Hg

Mean/S.E.M.

| Orbit \ Type | d | p | t |
|--------------|-------------|-------------|-------------|
| 0 | 8.90/2.632 | 8.63/2.632 | 6.95/2.279 |
| 1 | 10.10/2.632 | 16.40/2.632 | 19.28/2.279 |
| | 9.50/1.861 | 12.52/1.861 | 13.11/1.612 |

orbit p = 0.0022

omitting bag number 56:
Mean/S.E.M.

| Orbit \ Type | d | p | t | |
|--------------|-------------|-------------|-------------|-------------|
| 0 | 8.90/2.661 | 8.63/2.661 | 6.95/2.305 | 8.04/1.458 |
| 1 | 10.10/2.661 | 16.40/2.661 | 18.17/2.279 | 14.89/1.536 |
| | 9.50/1.882 | 12.52/1.882 | 11.76/1.742 | |

orbit p = 0.0065

V3 = PO₂ - mm Hg

Mean/S.E.M.

| Orbit \ Type | d | p | t | |
|--------------|--------------|--------------|--------------|--------------|
| 0 | 202.13/7.630 | 207.70/7.630 | 209.83/6.607 | 206.88/4.179 |
| 1 | 188.07/7.630 | 187.73/7.630 | 178.42/6.607 | 184.11/4.179 |
| | 195.10/5.395 | 197.72/5.395 | 194.13/4.672 | |

orbit p = 0.0018

omitting bag number 56:
Mean/S.E.M.

| Orbit \ Type | d | p | t | |
|--------------|--------------|--------------|--------------|--------------|
| 0 | 202.13/7.761 | 207.70/7.761 | 209.83/6.721 | 206.88/4.251 |
| 1 | 188.07/7.761 | 187.73/7.761 | 175.60/7.761 | 183.80/4.481 |
| | 195.10/5.488 | 197.72/5.488 | 195.16/5.081 | |

orbit p = 0.0025

Jacobson 4

| <u>Sample</u> | <u>Bag</u> | <u>Dewar</u> | <u>Orbit=1</u> | <u>Type</u> | <u>V1</u> |
|---------------|------------|--------------|----------------|-------------|-----------|
| 81 | 13 | e | 0 | t | 10.0 |
| 82 | 1 | g | 0 | d | 7.0 |
| 83 | 6 | a | 1 | d | 7.0 |
| 84 | 19 | f | 0 | p | 8.0 |
| 85 | 17 | b | 1 | p | 7.0 |
| 86 | 14 | c | 1 | t | 10.0 |
| 87 | 8 | b | 1 | t | 10.0 |
| 88 | 15 | e | 0 | p | 8.0 |
| 89 | 9 | g | 0 | t | 10.0 |
| 90 | 3 | c | 1 | d | 8.0 |
| 91 | 20 | a | 1 | p | 8.0 |
| 92 | 2 | f | 0 | d | 10.0 |
| 93 | 5 | b | 1 | d | 10.0 |
| 94 | 10 | a | 1 | t | 14.0 |
| 95 | 21 | c | 1 | p | 10.0 |
| 96 | 16 | g | 0 | p | 10.0 |
| 97 | 11 | f | 0 | t | 10.0 |
| 98 | 4 | e | 0 | d | 7.0 |

V1 = Plasma Hemoglobin mg/dl

Mean/S.E.M.

| <u>Orbit \ Type</u> | <u>d</u> | <u>p</u> | <u>t</u> | |
|---------------------|------------|------------|-------------|------------|
| 0 | 8.00/0.892 | 8.67/0.892 | 10.00/0.892 | 8.89/0.515 |
| 1 | 8.33/0.892 | 8.33/0.892 | 11.33/0.892 | 9.33/0.515 |
| | 8.17/0.631 | 8.50/0.631 | 10.67/0.631 | |

type p = 0.0324

Jacobson 5 - Red Blood Cells

| Sample | Bag | Dewar | Orbit=1 | Type | V1 | V2 | V3 | V4 | V5 |
|--------|-----|-------|---------|------|-----|-----|-------|------|-------|
| 61 | 1 | g | 0 | d | 4.1 | 3.1 | 306.7 | 38.6 | 111.0 |
| 62 | 14 | c | 1 | t | 3.8 | 1.8 | 310.7 | 38.2 | 111.0 |
| 63 | 6 | a | 1 | d | 4.2 | 3.1 | 323.5 | 38.5 | 112.0 |
| 64 | 19 | f | 0 | p | 3.8 | 3.1 | 308.0 | 38.3 | 112.0 |
| 65 | 13 | e | 0 | t | 3.8 | 3.8 | 304.5 | 38.5 | 112.0 |
| 66 | 17 | b | 1 | p | 3.8 | 2.5 | 313.5 | 38.4 | 112.0 |
| 67 | 3 | c | 1 | d | 3.8 | 3.1 | 312.7 | 38.1 | 112.0 |
| 68 | 2 | f | 0 | d | 3.8 | 4.9 | 298.0 | 38.6 | 112.0 |
| 69 | 20 | a | 1 | p | 3.8 | 3.8 | 304.7 | 38.3 | 112.0 |
| 70 | 8 | b | 1 | t | 3.5 | 1.9 | 307.0 | 38.0 | 111.0 |
| 71 | 13 | e | 0 | t | 3.8 | 2.5 | 302.0 | 38.5 | 112.0 |
| 72 | 15 | e | 0 | p | 3.5 | 4.4 | 300.0 | 38.5 | 112.0 |
| 73 | 16 | g | 0 | p | 3.8 | 1.9 | 302.0 | 38.2 | 111.0 |
| 74 | 5 | b | 1 | d | 3.8 | 2.5 | 298.7 | 38.5 | 112.0 |
| 75 | 10 | a | 1 | t | 3.8 | 3.8 | 305.0 | 38.2 | 111.0 |
| 76 | 11 | f | 0 | t | 3.4 | 1.9 | 321.3 | 38.2 | 111.0 |
| 77 | 4 | e | 0 | d | 4.2 | 3.1 | 337.0 | 38.1 | 112.0 |
| 78 | 21 | c | 1 | p | 4.2 | 3.1 | 317.0 | 38.2 | 111.0 |

V1 = ATP μ moles / gm hgb

Mean/S.E.M.

| Orbit \ Type | d | p | t | |
|--------------|------------|------------|------------|------------|
| 0 | 4.03/0.121 | 3.70/0.121 | 3.67/0.121 | 3.80/0.070 |
| 1 | 3.93/0.121 | 3.93/0.121 | 3.70/0.121 | 3.86/0.070 |
| | 3.98/0.086 | 3.82/0.086 | 3.68/0.086 | |

V2 = 2,3DPG μ moles / gm hgb

Mean/S.E.M.

| Orbit \ Type | d | p | t | |
|--------------|------------|------------|------------|------------|
| 0 | 3.70/0.548 | 3.13/0.548 | 2.73/0.548 | 3.19/0.316 |
| 1 | 2.90/0.548 | 3.13/0.548 | 2.50/0.548 | 2.84/0.316 |
| | 3.30/0.387 | 3.13/0.387 | 2.62/0.387 | |

V3 = Glucose mg/dl

Mean/S.E.M.

| Orbit \ Type | d | p | t | |
|--------------|--------------|--------------|--------------|--------------|
| 0 | 313.90/6.454 | 303.33/6.454 | 309.27/6.454 | 308.83/3.725 |
| 1 | 311.63/6.454 | 311.73/6.454 | 307.57/6.454 | 310.31/3.725 |
| | 312.77/4.564 | 307.53/4.564 | 308.42/4.564 | |

(Continued next page)

V4 = Potassium mlq/liter

Mean/S.E.M.

| Orbit \ Type | d | p | t | |
|--------------|-------------|-------------|-------------|-------------|
| 0 | 38.43/0.109 | 38.33/0.109 | 38.40/0.109 | 38.39/0.063 |
| 1 | 38.37/0.109 | 38.30/0.109 | 38.13/0.109 | 38.27/0.063 |
| | 38.40/0.077 | 38.32/0.077 | 38.27/0.077 | |

V5 = Sodium mlq/liter

Mean/S.E.M.

| Orbit \ Type | d | p | t | |
|--------------|--------------|--------------|--------------|--------------|
| 0 | 111.67/0.272 | 111.67/0.272 | 111.67/0.272 | 111.67/0.157 |
| 1 | 112.00/0.272 | 111.67/0.272 | 111.00/0.272 | 111.56/0.157 |
| | 111.83/0.192 | 111.67/0.192 | 111.33/0.192 | |

Jacobson 6 - Red Blood Cells

| Sample | Bag | Dewar | Orbit=1 | Type | V1 | V2 | V3 |
|--------|-----|-------|---------|------|------|------|-------|
| 81 | 13 | e | 0 | t | 10.0 | 12.0 | 167.0 |
| 82 | 1 | g | 0 | d | 7.0 | 11.7 | 167.0 |
| 83 | 6 | a | 1 | d | 7.0 | 12.7 | 168.0 |
| 84 | 19 | f | 0 | p | 8.0 | 11.7 | 167.0 |
| 85 | 17 | b | 1 | p | 7.0 | 12.2 | 168.0 |
| 86 | 14 | c | 1 | t | 10.0 | 11.8 | 169.0 |
| 87 | 8 | b | 1 | t | 10.0 | 12.8 | 166.5 |
| 88 | 15 | e | 0 | p | 8.0 | 11.8 | 168.0 |
| 89 | 9 | g | 0 | t | 10.0 | 11.8 | 169.0 |
| 90 | 3 | c | 1 | d | 8.0 | 12.1 | 168.0 |
| 91 | 20 | a | 1 | p | 8.0 | 12.0 | 169.0 |
| 92 | 2 | f | 0 | d | 10.0 | 11.7 | 169.0 |
| 93 | 5 | b | 1 | d | 10.0 | 11.9 | 169.0 |
| 94 | 10 | a | 1 | t | 14.0 | 12.3 | 168.0 |
| 95 | 21 | c | 1 | p | 10.0 | 12.6 | 168.0 |
| 96 | 16 | g | 0 | p | 10.0 | 12.5 | 169.0 |
| 97 | 11 | f | 0 | t | 10.0 | 12.6 | 169.0 |
| 98 | 4 | e | 0 | d | 7.0 | 12.5 | 169.0 |

V1 = Plasma Hemoglobin mgs %

Mean/S.E.M.

| Orbit \ Type | d | p | t |
|--------------|------------|------------|-------------|
| 0 | 8.00/0.892 | 8.67/0.892 | 10.00/0.892 |
| 1 | 8.33/0.892 | 8.33/0.892 | 11.33/0.892 |
| | 8.17/0.631 | 8.50/0.631 | 10.67/0.631 |

type p = 0.0324

V2 = Plasma Potassium mlq/liter

Mean/S.E.M.

| Orbit \ Type | d | p | t |
|--------------|-------------|-------------|-------------|
| 0 | 11.97/0.246 | 12.00/0.246 | 12.13/0.246 |
| 1 | 12.23/0.246 | 12.27/0.246 | 12.30/0.246 |
| | 12.10/0.174 | 12.13/0.174 | 12.22/0.174 |

V3 = Plasma Sodium mlq/liter

Mean/S.E.M.

| Orbit \ Type | d | p | t |
|--------------|--------------|--------------|--------------|
| 0 | 168.33/0.573 | 168.00/0.573 | 168.33/0.573 |
| 1 | 168.33/0.573 | 168.33/0.573 | 167.83/0.573 |
| | 168.33/0.405 | 168.17/0.405 | 168.08/0.405 |

Jacobson 13

Red Cells Phospholipids and Cholesterol

| Sample | Bag | Dewar | Orbit=1 | Type | V1 | V2 |
|--------|-----|-------|---------|------|-------|-------|
| 21 | 14 | c | 1 | t | lost | lost |
| 22 | 17 | b | 1 | p | 21.84 | 11.99 |
| 23 | 6 | a | 1 | d | 21.20 | 11.65 |
| 24 | 13 | e | 0 | t | 22.01 | 11.72 |
| 25 | 1 | g | 0 | d | 22.25 | 10.99 |
| 26 | 19 | f | 0 | p | 22.46 | 10.97 |
| 27 | 3 | c | 1 | d | 21.43 | 10.94 |
| 28 | 15 | e | 0 | p | 23.42 | 13.82 |
| 29 | 8 | b | 1 | t | 23.05 | 13.16 |
| 30 | 9 | g | 0 | t | 22.40 | 12.65 |
| 31 | 20 | a | 1 | p | 21.87 | 12.38 |
| 32 | 2 | f | 0 | d | 24.45 | 12.83 |
| 33 | 4 | e | 0 | d | 23.17 | 13.32 |
| 34 | 16 | g | 0 | p | 26.09 | 13.32 |
| 35 | 11 | f | 0 | t | 24.43 | 11.51 |
| 36 | 10 | a | 1 | t | 26.68 | 12.04 |
| 37 | 21 | c | 1 | p | 21.00 | 12.21 |
| 38 | 5 | b | 1 | d | 21.96 | 11.02 |

V1 = Phospholipids $\mu\text{gP}/10^8$ cells

Mean/S.E.M.

| Orbit \ Type | d | p | t |
|--------------|-------------|-------------|-------------|
| 0 | 23.29/0.784 | 23.99/0.784 | 22.95/0.784 |
| 1 | 21.53/0.784 | 21.57/0.784 | 24.87/0.960 |
| | 22.41/0.554 | 22.79/0.554 | 23.71/0.607 |

V2 = Cholesterol $\mu\text{g} / 10^8$ cells

Mean/S.E.M.

| Orbit \ Type | d | p | t |
|--------------|-------------|-------------|-------------|
| 0 | 12.38/0.533 | 12.70/0.533 | 11.96/0.533 |
| 1 | 11.20/0.533 | 12.19/0.533 | 12.60/0.653 |
| | 11.79/0.377 | 12.45/0.377 | 12.22/0.413 |

Jacobson 14

Plasma Phospholipids and Cholesterol

| Sample | Bag | Dewar | Orbit=1 | Type | V1 | V2 |
|--------|-----|-------|---------|------|------|------|
| 1 | 13 | e | 0 | t | 1.18 | 1.48 |
| 2 | 17 | b | 1 | p | 1.19 | 1.45 |
| 3 | 19 | f | 0 | p | 1.27 | 1.43 |
| 4 | 1 | g | 0 | d | 1.22 | 1.37 |
| 5 | 6 | a | 1 | d | 1.24 | 1.46 |
| 6 | 14 | c | 1 | t | 1.20 | 1.42 |
| 7 | 3 | c | 1 | d | 1.20 | 1.45 |
| 8 | 9 | g | 0 | t | 1.20 | 1.51 |
| 9 | 8 | b | 1 | t | 1.19 | 1.51 |
| 10 | 2 | f | 0 | d | 1.25 | 1.52 |
| 11 | 15 | e | 0 | p | 1.37 | 1.34 |
| 12 | 20 | a | 1 | p | 1.50 | 1.36 |
| 13 | 4 | e | 0 | d | 1.45 | 1.31 |
| 14 | 16 | g | 0 | p | 1.33 | 1.19 |
| 15 | 5 | b | 1 | d | 1.27 | 1.31 |
| 16 | 11 | f | 0 | t | 1.36 | 1.43 |
| 17 | 21 | c | 1 | p | 1.19 | 1.23 |
| 18 | 10 | a | 1 | t | 1.33 | 1.31 |

V1 = Phospholipids $\mu\text{gP}/10^8$ cells

Mean/S.E.M.

| Orbit \ Type | d | p | t |
|--------------|------------|------------|------------|
| 0 | 1.31/0.061 | 1.32/0.061 | 1.25/0.061 |
| 1 | 1.24/0.061 | 1.29/0.061 | 1.24/0.061 |
| | 1.27/0.043 | 1.31/0.043 | 1.24/0.043 |

V2 = Cholesterol $\mu\text{g} / 10^8$ cells

Mean/S.E.M.

| Orbit \ Type | d | p | t |
|--------------|------------|------------|------------|
| 0 | 1.40/0.056 | 1.32/0.056 | 1.47/0.056 |
| 1 | 1.41/0.056 | 1.35/0.056 | 1.41/0.056 |
| | 1.40/0.040 | 1.33/0.040 | 1.44/0.040 |

Jacobson 15 Plasticizer DEHP

| <u>Sample</u> | <u>Bag</u> | <u>Dewar</u> | <u>Orbit=1</u> | <u>Type</u> | <u>V1</u> |
|---------------|------------|--------------|----------------|-------------|-----------|
| 4 | 1 | g | 0 | d | 4.65 |
| 5 | 6 | a | 1 | d | 4.30 |
| 7 | 3 | c | 1 | d | 4.45 |
| 10 | 2 | f | 0 | d | 4.25 |
| 13 | 4 | e | 0 | d | 4.90 |
| 15 | 5 | b | 1 | d | 4.85 |

V1 = Plasticizer mg/100ml plasma

Mean (Standard Error of the Mean)

Orbit

0 : 4.60 (.177) 1 : 4.53 (.177)

Kenney 1 Surface Proteins - Platelets

| Sample | Bag | Dewar | Orbit=1 | Type | V1 | V2 | V3 |
|--------|-----|-------|---------|------|------|------|------|
| 500 | 50 | h | 0 | t | 5.8 | 5.8 | 5.9 |
| 501 | 60 | h | 0 | p | 5.5 | 5.7 | 6.0 |
| 502 | 56 | d | 1 | t | 2.2 | 3.2 | 2.7 |
| 503 | 61 | d | 1 | p | 3.5 | 2.0 | 2.9 |
| 504 | 48 | d | 1 | d | 10.8 | 10.1 | 9.9 |
| 505 | 45 | h | 0 | d | 10.8 | 9.8 | 11.0 |
| 506 | 59 | h | 0 | p | 8.5 | 9.4 | 9.4 |
| 507 | 43 | d | 1 | d | 10.3 | 10.1 | 9.5 |
| 508 | 54 | d | 1 | t | 1.0 | 1.0 | 1.5 |
| 509 | 62 | d | 1 | p | 4.5 | 3.8 | 3.0 |
| 510 | 47 | h | 0 | d | 7.7 | 10.0 | 9.9 |
| 511 | 55 | h | 0 | t | 7.3 | 6.7 | 6.3 |

V1 = ^{125}I - Membrane Proteins

Mean/S.E.M.

| Orbit \ Type | d | p | t | |
|--------------|-------------|------------|------------|------------|
| 0 | 9.25/1.419 | 7.00/1.419 | 6.55/1.419 | 7.60/0.819 |
| 1 | 10.55/1.419 | 4.00/1.419 | 1.60/1.419 | 5.38/0.819 |
| | 9.90/1.004 | 5.50/1.004 | 4.07/1.004 | |

type p = 0.0085

omitting bag number 56:

Mean/S.E.M.

| Orbit \ Type | d | p | t | |
|--------------|-------------|------------|------------|------------|
| 0 | 9.25/1.051 | 7.00/1.051 | 6.55/1.051 | 7.60/0.607 |
| 1 | 10.55/1.051 | 4.00/1.051 | 1.00/1.487 | 6.02/0.665 |
| | 9.90/0.743 | 5.50/0.743 | 4.70/0.858 | |

type p = 0.0088

V2 = ^3H - Membrane Glycoproteins

Mean/S.E.M.

| Orbit \ Type | d | p | t | |
|--------------|-------------|------------|------------|------------|
| 0 | 9.90/1.263 | 7.55/1.263 | 6.25/1.263 | 7.90/0.729 |
| 1 | 10.10/1.263 | 2.90/1.263 | 2.10/1.263 | 5.03/0.729 |
| | 10.00/0.893 | 5.23/0.893 | 4.17/0.893 | |

type p = 0.0038

orbit p = 0.0240

omitting bag number 56:
Mean/S.E.M.

| Orbit \ Type | d | p | t | |
|--------------|-------------|------------|------------|------------|
| 0 | 9.90/0.943 | 7.55/0.943 | 6.25/0.943 | 7.90/0.544 |
| 1 | 10.10/0.943 | 2.90/0.943 | 1.00/1.333 | 5.40/0.596 |
| | 10.00/0.667 | 5.23/0.667 | 4.50/0.770 | |

type p = 0.0038
orbit p = 0.0270

V3 = Cytoskeleton

Mean/S.E.M.

| Orbit \ Type | d | p | t | |
|--------------|-------------|------------|------------|------------|
| 0 | 10.45/1.010 | 7.70/1.010 | 6.10/1.010 | 8.08/0.583 |
| 1 | 9.70/1.010 | 2.95/1.010 | 2.10/1.010 | 4.92/0.583 |
| | 10.08/0.714 | 5.33/0.714 | 4.10/0.714 | |

type p = 0.0008
orbit p = 0.0049

omitting bag number 56:
Mean/S.E.M.

| Orbit \ Type | d | p | t | |
|--------------|-------------|------------|------------|------------|
| 0 | 10.45/0.809 | 7.70/0.809 | 6.10/0.809 | 8.08/0.467 |
| 1 | 9.70/0.809 | 2.95/0.809 | 1.50/1.145 | 5.36/0.512 |
| | 10.08/0.572 | 5.33/0.572 | 4.57/0.661 | |

type p = 0.0019
orbit p = 0.0111

Kevy 1 - Red Cell Osmotic Fragility

| <u>Sample</u> | <u>Baq</u> | <u>Dewar</u> | <u>Orbit=1</u> | <u>Type</u> | <u>V1</u> | <u>V2</u> | <u>V3</u> |
|---------------|------------|--------------|----------------|-------------|-----------|-----------|-----------|
| 121 | 19 | f | 0 | p | 2.85 | 7.14 | 27.14 |
| 122 | 14 | c | 1 | t | 2.94 | 7.35 | 26.47 |
| 123 | 13 | e | 0 | t | 1.40 | 5.63 | 22.53 |
| 124 | 17 | b | 1 | p | 2.63 | 7.89 | 28.94 |
| 125 | 1 | g | 0 | d | 2.85 | 8.57 | 31.42 |
| 126 | 6 | a | 1 | d | 4.34 | 7.24 | 27.53 |
| 127 | 2 | f | 0 | d | 2.94 | 4.41 | 35.29 |
| 128 | 9 | g | 0 | t | 4.34 | 7.24 | 28.98 |
| 129 | 3 | c | 1 | d | 2.85 | 7.14 | 34.28 |
| 130 | 8 | b | 1 | t | 4.41 | 7.35 | 32.35 |
| 131 | 15 | e | 0 | p | 1.47 | 8.23 | 36.76 |
| 132 | 20 | a | 1 | p | 1.47 | 4.41 | 32.35 |
| 133 | 10 | a | 1 | t | 8.57 | 10.00 | 45.71 |
| 134 | 5 | b | 1 | d | 6.84 | 10.95 | 38.35 |
| 135 | 16 | g | 0 | p | 2.89 | 7.24 | 36.23 |
| 136 | 4 | e | 0 | d | 0.59 | 5.97 | 32.83 |
| 137 | 21 | c | 1 | p | 1.49 | 2.98 | 29.85 |
| 138 | 11 | f | 0 | t | 1.21 | 1.51 | 28.78 |

V1 = Percent Hemolysis, 0.6% Saline

Mean/S.E.M.

| <u>Orbit \ Type</u> | <u>d</u> | <u>p</u> | <u>t</u> | |
|---------------------|------------|------------|------------|------------|
| 0 | 2.13/1.015 | 2.40/1.015 | 2.32/1.015 | 2.28/0.586 |
| 1 | 4.68/1.015 | 1.86/1.015 | 5.31/1.015 | 3.95/0.586 |
| | 3.40/0.718 | 2.13/0.718 | 3.81/0.718 | |

V2 = Percent Hemolysis, 0.55% Saline

Mean/S.E.M.

| <u>Orbit \ Type</u> | <u>d</u> | <u>p</u> | <u>t</u> | |
|---------------------|------------|------------|------------|------------|
| 0 | 6.32/1.224 | 7.54/1.224 | 4.79/1.224 | 6.22/0.706 |
| 1 | 8.44/1.224 | 5.09/1.224 | 8.23/1.224 | 7.26/0.706 |
| | 7.38/0.865 | 6.32/0.865 | 6.51/0.865 | |

V3 = Percent Hemolysis, 0.5% Saline

Mean/S.E.M.

| <u>Orbit \ Type</u> | <u>d</u> | <u>p</u> | <u>t</u> | |
|---------------------|-------------|-------------|-------------|-------------|
| 0 | 33.18/3.133 | 33.38/3.133 | 26.76/3.133 | 31.11/1.809 |
| 1 | 33.39/3.133 | 30.38/3.133 | 34.84/3.133 | 32.87/1.809 |
| | 33.28/2.216 | 31.88/2.216 | 30.80/2.216 | |

Lionetti 1 - White Cells Recovery and Viability

| Sample | Bag | Dewar | Orbit=1 | Type | V1 | V2 | V3 |
|--------|-----|-------|---------|------|------|------|----|
| 801 | 22 | a | 1 | d | 9.7 | 52.9 | 89 |
| 802 | 35 | b | 1 | t | 12.1 | 66.0 | 83 |
| 803 | 31 | g | 0 | t | 9.3 | 50.7 | 84 |
| 804 | 24 | f | 0 | d | 8.3 | 45.3 | 82 |
| 805 | 39 | c | 1 | p | 11.8 | 64.4 | 90 |
| 806 | 40 | e | 0 | p | 9.1 | 49.6 | 93 |
| 807 | 25 | g | 0 | d | 13.6 | 74.2 | 12 |
| 808 | 32 | e | 0 | t | 11.0 | 60.0 | 79 |
| 809 | 29 | a | 1 | t | 10.6 | 57.8 | 85 |
| 810 | 38 | f | 0 | p | 11.3 | 61.6 | 89 |
| 811 | 41 | b | 1 | p | 8.0 | 43.6 | 77 |
| 812 | 23 | c | 1 | d | 12.3 | 67.1 | 88 |
| 813 | 37 | a | 1 | p | 10.7 | 58.4 | 80 |
| 814 | 28 | e | 0 | d | 12.4 | 67.7 | 67 |
| 815 | 33 | f | 0 | t | 11.9 | 64.9 | 77 |
| 816 | 34 | c | 1 | t | 11.3 | 61.6 | 56 |
| 817 | 36 | g | 0 | p | 12.8 | 69.8 | 78 |
| 818 | 26 | b | 1 | d | 9.6 | 52.4 | 73 |

V1 = count $\times 10^6$ cells / ml - not analysed

V2 = % recovery

Mean/S.E.M.

| Orbit \ Type | d | p | t |
|--------------|-------------|-------------|-------------|
| 0 | 62.40/5.707 | 60.33/5.707 | 58.53/5.707 |
| 1 | 57.47/5.707 | 55.47/5.707 | 61.80/5.707 |
| | 59.93/4.035 | 57.90/4.035 | 60.17/4.035 |

V3 = viability % FDA positive

Mean/S.E.M.

| Orbit \ Type | d | p | t |
|--------------|--------------|--------------|--------------|
| 0 | 53.67/10.057 | 86.67/10.057 | 80.00/10.057 |
| 1 | 83.33/10.057 | 82.33/10.057 | 74.67/10.057 |
| | 68.50/7.112 | 84.50/7.112 | 77.33/7.112 |

Lionetti 2 - White Cells Phagocytic Index

| Sample | Bag | Dewar | Orbit=1 | Type | V1 | V2 |
|--------|-----|-------|---------|------|-----|-----|
| 801 | 22 | a | 1 | d | 147 | 141 |
| 802 | 35 | b | 1 | t | 142 | 143 |
| 803 | 31 | g | 0 | t | 175 | 195 |
| 804 | 24 | f | 0 | d | 171 | 184 |
| 805 | 39 | c | 1 | p | 143 | 146 |
| 806 | 40 | e | 0 | p | 159 | 178 |
| 807 | 25 | g | 0 | d | 147 | 174 |
| 808 | 32 | e | 0 | t | 157 | 148 |
| 809 | 29 | a | 1 | t | 147 | 154 |
| 810 | 38 | f | 0 | p | 148 | 133 |
| 811 | 41 | b | 1 | p | 155 | 178 |
| 812 | 23 | c | 1 | d | 123 | 130 |
| 813 | 37 | a | 1 | p | 141 | 142 |
| 814 | 28 | e | 0 | d | 156 | 116 |
| 815 | 33 | f | 0 | t | 147 | 154 |
| 816 | 34 | c | 1 | t | 163 | 170 |
| 817 | 36 | g | 0 | p | 155 | 162 |
| 818 | 26 | b | 1 | d | 149 | 149 |

V1 = Ingestion of ^{125}I Staph A, % of N-Ethylmaleimide treated controls, determination 1

Mean/S.E.M.

| Orbit \ Type | d | p | t |
|--------------|--------------|--------------|--------------|
| 0 | 158.00/6.525 | 154.00/6.525 | 159.67/6.525 |
| 1 | 139.67/6.525 | 146.33/6.525 | 150.67/6.525 |
| | 148.83/4.614 | 150.17/4.614 | 155.17/4.614 |

orbit p = 0.0490

V2 = Ingestion of ^{125}I Staph A, % of N-Ethylmaleimide treated controls, determination 2

Mean/S.E.M.

| Orbit \ Type | d | p | t |
|--------------|---------------|---------------|---------------|
| 0 | 158.00/13.307 | 157.67/13.307 | 165.67/13.307 |
| 1 | 140.00/13.307 | 155.33/13.307 | 155.67/13.307 |
| | 149.00/9.408 | 156.50/9.408 | 160.67/9.408 |

Lionetti 3 - White Cell Glucose Oxidation

| Sample | Bag | Dewar | Orbit=1 | Type | V1 | V2 |
|--------|-----|-------|---------|------|-----|-----|
| 801 | 22 | a | 1 | d | 260 | 90 |
| 802 | 35 | b | 1 | t | 290 | 90 |
| 803 | 31 | g | 0 | t | 320 | 110 |
| 804 | 24 | f | 0 | d | 320 | 120 |
| 805 | 39 | c | 1 | p | 280 | 100 |
| 806 | 40 | e | 0 | p | 410 | 120 |
| 807 | 25 | g | 0 | d | 190 | 80 |
| 808 | 32 | e | 0 | t | 340 | 130 |
| 809 | 29 | a | 1 | t | 290 | 100 |
| 810 | 38 | f | 0 | p | 310 | 120 |
| 811 | 41 | b | 1 | p | 390 | 110 |
| 812 | 23 | c | 1 | d | 200 | 60 |
| 813 | 37 | a | 1 | p | 320 | 100 |
| 814 | 28 | e | 0 | d | 260 | 90 |
| 815 | 33 | f | 0 | t | 360 | 120 |
| 816 | 34 | c | 1 | t | 440 | 90 |
| 817 | 36 | g | 0 | p | 370 | 110 |
| 818 | 26 | b | 1 | d | 280 | 90 |

V1 = ^{14}C Glucose Oxidation, Stimulated by PMA, % over background

Mean/S.E.M.

| Orbit \ Type | d | p | t |
|--------------|---------------|---------------|-----------------------------|
| 0 | 256.67/32.914 | 363.33/32.914 | 340.00/32.914 320.00/19.003 |
| 1 | 246.67/32.914 | 330.00/32.914 | 340.00/32.914 305.56/19.003 |
| | 251.67/23.274 | 346.67/23.274 | 340.00/23.274 |

type p = 0.0237

V2 = ^{14}C Glucose Oxidation, Stimulated by FMLP, % over background

Mean/S.E.M.

| Orbit \ Type | d | p | t |
|--------------|-------------|--------------|---------------------------|
| 0 | 96.67/7.201 | 116.67/7.201 | 120.00/7.201 111.11/4.157 |
| 1 | 80.00/7.201 | 103.33/7.201 | 93.33/7.201 92.22/4.157 |
| | 88.33/5.092 | 110.00/5.092 | 106.67/5.092 |

type p = 0.0230

orbit p = 0.0075

Szymanski 1 - Red Cells Agglutination

| Sample | Bag | Dewar | Orbit=1 | Type | V1 | V2 | V3 | V4 |
|--------|-----|-------|---------|------|-------|-------|------|--------|
| 201 | 19 | f | 0 | p | 14.08 | 106.8 | 8.35 | 115.75 |
| 202 | 6 | a | 1 | d | 14.20 | 72.5 | 8.58 | 116.25 |
| 203 | 17 | b | 1 | p | 14.50 | 73.8 | 8.58 | 116.25 |
| 204 | 14 | c | 1 | t | 14.18 | 69.0 | 7.95 | 123.50 |
| 205 | 1 | g | 0 | d | 14.48 | 73.0 | 6.78 | 103.67 |
| 206 | 13 | e | 0 | t | 13.95 | 65.2 | 6.93 | 110.33 |
| 207 | 2 | f | 0 | d | 14.20 | 56.3 | 6.73 | 99.50 |
| 208 | 3 | c | 1 | d | 12.28 | 74.4 | 7.28 | 98.67 |
| 209 | 9 | g | 0 | t | 14.33 | 64.6 | 7.13 | 96.25 |
| 210 | 20 | a | 1 | p | 14.70 | 58.1 | 7.85 | 94.50 |
| 211 | 8 | b | 1 | t | 13.83 | 63.0 | 8.93 | 99.75 |
| 212 | 15 | e | 0 | p | 14.38 | 53.9 | 8.63 | 99.00 |
| 213 | 4 | e | 0 | d | 14.90 | 60.5 | 9.13 | 99.25 |
| 214 | 11 | f | 0 | t | 16.40 | 71.8 | 9.25 | 94.75 |
| 215 | 21 | c | 1 | p | 16.25 | 75.2 | 9.25 | 94.25 |
| 216 | 5 | b | 1 | d | 16.63 | 69.4 | 8.85 | 93.25 |
| 217 | 16 | g | 0 | p | 16.30 | 62.2 | 9.15 | 96.50 |
| 218 | 10 | a | 1 | t | 16.60 | 76.3 | 8.68 | 91.00 |

V1 = IgG on intact cells, molecules / cell

Mean/S.E.M.

| Orbit \ Type | d | p | t | |
|--------------|-------------|-------------|-------------|-------------|
| 0 | 14.53/0.791 | 14.92/0.791 | 14.89/0.791 | 14.78/0.457 |
| 1 | 14.37/0.791 | 15.15/0.791 | 14.87/0.791 | 14.80/0.457 |
| | 14.45/0.559 | 15.04/0.559 | 14.88/0.559 | |

V2 = IgG inside cells, molecules/cell

Mean/S.E.M.

| Orbit \ Type | d | p | t | |
|--------------|-------------|-------------|-------------|-------------|
| 0 | 63.27/7.607 | 74.30/7.607 | 67.20/7.607 | 68.26/4.392 |
| 1 | 72.10/7.607 | 69.03/7.607 | 69.43/7.607 | 70.19/4.392 |
| | 67.68/5.379 | 71.67/5.379 | 68.32/5.379 | |

V3 = C3c on intact cells, molecules/cell

Mean/S.E.M.

| Orbit \ Type | d | p | t | |
|--------------|------------|------------|------------|------------|
| 0 | 7.55/0.535 | 8.71/0.535 | 7.77/0.535 | 8.01/0.309 |
| 1 | 8.23/0.535 | 8.56/0.535 | 8.52/0.535 | 8.44/0.309 |
| | 7.89/0.378 | 8.64/0.378 | 8.15/0.378 | |

V4 = C3d on intact cells, molecules/cell

Mean/S.E.M.

| Orbit \ Type | d | p | t | |
|--------------|--------------|--------------|--------------|--------------|
| 0 | 100.81/6.568 | 103.75/6.568 | 100.44/6.568 | 101.67/3.792 |
| 1 | 102.72/6.568 | 101.67/6.568 | 104.75/6.568 | 103.05/3.792 |
| | 101.77/4.644 | 102.71/4.644 | 102.60/4.644 | |

Mean (Standard Error of the Mean)

Type

d : 101.77 (3.20) p : 102.71 (4.26) t : 102.60 (4.97)

Orbit

0 : 101.67 (2.35) 1 : 103.05 (4.06)

1. The first part of the document is a list of names and addresses, which appears to be a directory or a list of contacts. The names are written in a cursive script, and the addresses are listed below them. The list includes names such as "John Smith", "Mary Jones", and "Robert Brown", among others. The addresses are also listed, often including street names and city names.

2. The second part of the document is a series of short, handwritten notes or entries. These notes are written in a cursive script and are organized into a list. The notes appear to be related to the names and addresses listed in the first part, possibly providing additional information or details about each contact.

3. The third part of the document is a series of short, handwritten notes or entries, similar to the second part. These notes are also written in a cursive script and are organized into a list. The notes appear to be related to the names and addresses listed in the first part, possibly providing additional information or details about each contact.

4. The fourth part of the document is a series of short, handwritten notes or entries, similar to the second and third parts. These notes are also written in a cursive script and are organized into a list. The notes appear to be related to the names and addresses listed in the first part, possibly providing additional information or details about each contact.

5. The fifth part of the document is a series of short, handwritten notes or entries, similar to the second, third, and fourth parts. These notes are also written in a cursive script and are organized into a list. The notes appear to be related to the names and addresses listed in the first part, possibly providing additional information or details about each contact.

Szymanski 2 - Red Cells Agglutination

| Sample | Bag | Dewar | Orbit=1 | Type | V1 | V2 | V3 | V4 |
|--------|-----|-------|---------|------|-------|-------|-------|-------|
| 201 | 19 | f | 0 | p | 21.90 | 48.85 | 76.30 | 54.85 |
| 202 | 6 | a | 1 | d | 21.25 | 47.10 | 73.10 | 50.45 |
| 203 | 17 | b | 1 | p | 18.25 | 44.30 | 73.30 | 50.55 |
| 204 | 14 | c | 1 | t | 16.30 | 40.40 | 72.95 | 50.45 |
| 205 | 1 | g | 0 | d | 22.25 | 57.40 | 77.25 | 56.35 |
| 206 | 13 | e | 0 | t | 21.35 | 48.60 | 76.00 | 55.70 |
| 207 | 2 | f | 0 | d | 21.60 | 46.95 | 74.60 | 54.70 |
| 208 | 3 | c | 1 | d | 22.20 | 48.30 | 75.35 | 53.60 |
| 209 | 9 | g | 0 | t | 14.35 | 39.00 | 73.00 | 49.65 |
| 210 | 20 | a | 1 | p | 16.80 | 42.20 | 72.80 | 49.60 |
| 211 | 8 | b | 1 | t | 27.85 | 54.90 | 78.00 | 56.15 |
| 212 | 15 | e | 0 | p | 22.10 | 50.15 | 77.40 | 56.80 |
| 213 | 4 | e | 0 | d | 26.55 | 54.85 | 77.80 | 56.95 |
| 214 | 11 | f | 0 | t | 23.85 | 50.75 | 77.25 | 56.80 |
| 215 | 21 | c | 1 | p | 26.50 | 52.55 | 76.95 | 56.00 |
| 216 | 5 | b | 1 | d | 24.60 | 51.30 | 76.55 | 56.90 |
| 217 | 16 | g | 0 | p | 26.30 | 51.65 | 76.90 | 56.60 |
| 218 | 10 | a | 1 | t | 20.90 | 48.15 | 76.20 | 54.40 |

V1 = % Agglutination with anti-C3a

Mean/S.E.M.

| Orbit \ Type | d | p | t |
|--------------|-------------|-------------|-------------|
| 0 | 23.47/2.378 | 23.43/2.378 | 19.85/2.378 |
| 1 | 22.68/2.378 | 20.52/2.378 | 21.68/2.378 |
| | 23.08/1.682 | 21.98/1.682 | 20.77/1.682 |

V2 = % Agglutination with anti-C3c

Mean/S.E.M.

| Orbit \ Type | d | p | t |
|--------------|-------------|-------------|-------------|
| 0 | 53.07/2.963 | 50.22/2.963 | 46.12/2.963 |
| 1 | 48.90/2.963 | 46.35/2.963 | 47.82/2.963 |
| | 50.98/2.095 | 48.28/2.095 | 46.97/2.095 |

V3 = % Agglutination with anti-C3d

Mean/S.E.M.

| Orbit \ Type | d | p | t |
|--------------|-------------|-------------|-------------|
| 0 | 76.55/1.124 | 76.87/1.124 | 75.42/1.124 |
| 1 | 75.00/1.124 | 74.35/1.124 | 75.72/1.124 |
| | 75.78/0.795 | 75.61/0.795 | 75.57/0.795 |

V4 = % Agglutination with anti-IgG

Mean/S.E.M.

| Orbit \ Type | d | p | t |
|--------------|-------------|-------------|-------------|
| 0 | 56.00/1.636 | 56.08/1.636 | 54.05/1.636 |
| 1 | 53.65/1.636 | 52.05/1.636 | 53.67/1.646 |
| | 54.83/1.157 | 54.07/1.157 | 53.86/1.157 |

Szymanski 3 - Platelets

| Sample | Bag | Dewar | Orbit=1 | Type | V1 | V2 | V3 |
|--------|-----|-------|---------|------|------|------|------|
| 600 | 60 | h | 0 | p | 4018 | 2085 | 2049 |
| 601 | 45 | h | 0 | d | 6611 | 2198 | 2123 |
| 602 | 50 | h | 0 | t | 1597 | 2041 | 2002 |
| 603 | 61 | d | 1 | p | 2840 | 1574 | 1840 |
| 604 | 48 | d | 1 | d | 6374 | 2181 | 2750 |
| 605 | 56 | d | 1 | t | 2997 | 1218 | 1200 |
| 606 | 54 | d | 1 | t | 1844 | 1148 | 1207 |
| 607 | 62 | d | 1 | p | 2488 | 1478 | 1934 |
| 608 | 43 | d | 1 | d | 5855 | 2312 | 2783 |
| 609 | 47 | h | 0 | d | 5425 | 2302 | 2560 |
| 610 | 59 | h | 0 | p | 5059 | 2164 | 2461 |
| 611 | 55 | h | 0 | t | 5902 | 2132 | 2400 |
| 612 | 46 | h | 0 | d | 5656 | 2030 | 2324 |
| 613 | 53 | d | 1 | t | 2147 | 1561 | 1719 |
| 614 | 63 | h | 0 | p | 4208 | 2013 | 2218 |
| 615 | 51 | h | 0 | t | 4355 | 1775 | 2412 |
| 616 | 58 | d | 1 | p | 5433 | 1715 | 2273 |
| 617 | 44 | d | 1 | d | 6586 | 2130 | 2775 |

V1 = Molecules of bound IgG/platelet

Mean/S.E.M.

| Orbit \ Type | d | p | t | |
|--------------|------------|------------|------------|------------|
| 0 | 5897/688.9 | 4428/688.9 | 3951/688.9 | 4759/397.8 |
| 1 | 6271/688.9 | 3587/688.9 | 2329/688.9 | 4062/397.8 |
| | 6084/487.2 | 4007/487.2 | 3140/487.2 | |

type p = 0.0032

omitting bag number 56:

Mean/S.E.M.

| Orbit \ Type | d | p | t | |
|--------------|------------|------------|------------|------------|
| 0 | 5897/705.4 | 4428/705.4 | 3951/705.4 | 4759/407.2 |
| 1 | 6271/705.4 | 3587/705.4 | 1995/863.9 | 4195/431.9 |
| | 6084/498.8 | 4007/498.8 | 3169/546.4 | |

type p = 0.0052

V2 = Number of C3d molecules/platelet

Mean/S.E.M.

| Orbit \ Type | d | p | t | |
|--------------|------------|------------|------------|------------|
| 0 | 2176/85.26 | 2087/85.26 | 1982/85.26 | 2082/49.22 |
| 1 | 2207/85.26 | 1589/85.26 | 1309/85.26 | 1701/49.22 |
| | 2192/60.29 | 1838/60.29 | 1645/60.29 | |

type p = 0.0001

orbit p = 0.0001

interaction p = 0.0037

omitting bag number 56:

Mean/S.E.M.

| Orbit \ Type | d | p | t | |
|--------------|------------|------------|------------|------------|
| 0 | 2176/86.91 | 2087/86.91 | 1982/86.91 | 2082/50.18 |
| 1 | 2207/86.91 | 1589/86.91 | 1354/106.4 | 1762/53.22 |
| | 2192/61.45 | 1838/61.45 | 1731/67.32 | |

type p = 0.0005

orbit p = 0.0011

interaction p = 0.0085

v3 = Number of C3c molecules/platelet

Mean/S.E.M.

| Orbit \ Type | d | p | t | |
|--------------|------------|------------|------------|------------|
| 0 | 2335/126.0 | 2242/126.0 | 2271/126.0 | 2283/72.77 |
| 1 | 2769/126.0 | 2015/126.0 | 1375/126.0 | 2053/72.77 |
| | 2552/89.12 | 2129/89.12 | 1823/89.12 | |

type p = 0.0003

orbit p = 0.0454

interaction p = 0.0007

omitting bag number 56:

Mean/S.E.M.

| Orbit \ Type | d | p | t | |
|--------------|------------|------------|------------|------------|
| 0 | 2335/126.2 | 2242/126.2 | 2271/126.2 | 2283/72.89 |
| 1 | 2769/126.2 | 2015/126.2 | 1463/154.6 | 2160/77.30 |
| | 2552/89.25 | 2129/89.25 | 1948/97.77 | |

type p = 0.0019

interaction p = 0.0025

Szymanski - Platelets

| Sample | Bag | Dewar | Orbit=1 | Type | V1 | V2 |
|--------|-----|-------|---------|------|-------|-------|
| 600 | 60 | h | 0 | p | 37122 | 33104 |
| 601 | 45 | h | 0 | d | 52517 | 45906 |
| 602 | 50 | h | 0 | t | 30479 | 29382 |
| 603 | 61 | d | 1 | p | 28264 | 25424 |
| 604 | 48 | d | 1 | d | 47284 | 40910 |
| 605 | 56 | d | 1 | t | 16298 | 13301 |
| 606 | 54 | d | 1 | t | 16234 | 14390 |
| 607 | 62 | d | 1 | p | 26533 | 24045 |
| 608 | 43 | d | 1 | d | 54000 | 48145 |
| 609 | 47 | h | 0 | d | 47809 | 42384 |
| 610 | 59 | h | 0 | p | 37094 | 32035 |
| 611 | 55 | h | 0 | t | 33935 | 28033 |
| 612 | 46 | h | 0 | d | 44638 | 38982 |
| 613 | 53 | d | 1 | t | 24738 | 22591 |
| 614 | 63 | h | 0 | p | 36985 | 32777 |
| 615 | 51 | h | 0 | t | 39813 | 35458 |
| 616 | 58 | d | 1 | p | 42508 | 36978 |
| 617 | 44 | d | 1 | d | 55841 | 49255 |

V1 Total Platelet IgG (molecules/platelet)

Mean/S.E.M.

| Orbit \ Type | d | p | t |
|--------------|------------|------------|------------|
| 0 | 48321/2972 | 37067/2972 | 34742/2972 |
| 1 | 52375/2972 | 32435/2972 | 19090/2972 |
| | 50348/2101 | 34751/2101 | 26916/2101 |

type p = 0.0001

orbit p = 0.0457

interaction p = 0.0200

omitting bag number 56:

Mean/S.E.M.

| Orbit \ Type | d | p | t |
|--------------|------------|------------|------------|
| 0 | 48321/3047 | 37067/3047 | 34742/3047 |
| 1 | 52375/3047 | 32435/3047 | 18490/3732 |
| | 50348/2154 | 34751/2154 | 29039/2360 |

type p = 0.0001

interaction p = 0.0342

V2 Total free IgG in Frozen - Thawed platelets (molecules/platelet)

Mean/S.E.M.

| Orbit \ Type | d | p | t | |
|--------------|------------|------------|------------|------------|
| 0 | 42424/2632 | 32629/2632 | 30957/2632 | 35340/1520 |
| 1 | 46103/2632 | 28816/2632 | 16761/2632 | 30560/1520 |
| | 44264/1861 | 30727/1861 | 23859/1861 | |

type p = 0.0001

orbit p = 0.0461

interaction p = 0.0171

omitting bag number 56:

Mean/S.E.M.

| Orbit \ Type | d | p | t | |
|--------------|------------|------------|------------|------------|
| 0 | 42424/2648 | 32629/2648 | 30957/2648 | 35340/1529 |
| 1 | 46103/2648 | 28816/2648 | 18491/3243 | 32717/1622 |
| | 44264/1873 | 30727/1873 | 25970/2051 | |

type p = 0.0001

interaction p = 0.0327

Taylor White Cells

| Sample | Bag | Dewar | Orbit=1 | Type | V1 | V2 | V3 | V4 | V5 | V6 | V7 | V8 |
|--------|-----|-------|---------|------|----|----|------|------|------|-------|------|------|
| 901 | 22 | a | 1 | d | 94 | 95 | 38.2 | 41.5 | 16.9 | 27.0 | 38.6 | . |
| 904 | 40 | e | 0 | p | 98 | 95 | 38.4 | 30.0 | 36.3 | 79.8 | 55.1 | 0.78 |
| 910 | 29 | a | 1 | t | 91 | 90 | 52.9 | 75.9 | 47.1 | 98.7 | 88.4 | 0.72 |
| 912 | 32 | e | 0 | t | 94 | 97 | 15.6 | 23.4 | 26.0 | 80.3 | 81.0 | 2.06 |
| 913 | 37 | a | 1 | p | 98 | 97 | 17.1 | 21.0 | 22.8 | 115.5 | 94.9 | 1.15 |
| 918 | 28 | e | 0 | d | 95 | 98 | 19.5 | 26.8 | 60.4 | 79.7 | 74.8 | 4.98 |

V1 = % viability

Mean/S.E.M.

| Orbit \ Type | d | p | t |
|--------------|-------------|-------------|-------------|
| 0 | 95.00/2.887 | 98.00/2.887 | 94.00/2.887 |
| 1 | 94.00/2.887 | 98.00/2.887 | 91.00/2.887 |
| | 94.50/2.041 | 98.00/2.041 | 92.50/2.041 |

V2 = % Viability - 72 hours

Mean/S.E.M.

| Orbit \ Type | d | p | t |
|--------------|-------------|-------------|-------------|
| 0 | 98.00/2.261 | 95.00/2.261 | 97.00/2.261 |
| 1 | 94.00/2.261 | 97.00/2.261 | 90.00/2.261 |
| | 96.50/1.985 | 96.00/1.985 | 93.50/1.985 |

V3 = Protein Synthesis w/o Mitogen

Mean/S.E.M.

| Orbit \ Type | d | p | t |
|--------------|-------------|-------------|-------------|
| 0 | 19.50/15.37 | 38.40/15.37 | 15.60/15.37 |
| 1 | 38.20/15.37 | 17.10/15.37 | 52.90/15.37 |
| | 28.85/10.87 | 27.75/10.87 | 34.25/10.87 |

V4 = Protein Synthesis in response to phytohemagglutinin

Mean/S.E.M.

| Orbit \ Type | d | p | t |
|--------------|-------------|-------------|-------------|
| 0 | 26.80/19.75 | 30.00/19.75 | 23.40/19.75 |
| 1 | 41.50/19.75 | 21.00/19.75 | 75.90/19.75 |
| | 34.15/13.97 | 25.50/13.97 | 49.65/13.97 |

(Continued on next page)

15 = Blastogenesis w/o Mitogen

Mean/S.E.M.

| Orbit \ Type | d | p | t | |
|--------------|-------------|-------------|-------------|-------------|
| 0 | 60.40/16.85 | 36.30/16.85 | 26.00/16.85 | 40.90/9.729 |
| 1 | 16.90/16.85 | 22.80/16.85 | 47.10/16.85 | 28.93/9.729 |
| | 38.65/11.92 | 29.55/11.92 | 53.86/11.92 | |

16 = Blastogenesis in response to PHA

Mean/S.E.M.

| Orbit \ Type | d | p | t | |
|--------------|-------------|-------------|-------------|-------------|
| 0 | 79.70/34.23 | 79.80/34.23 | 80.30/34.23 | 79.93/19.19 |
| 1 | 27.00/34.23 | 115.5/34.23 | 98.70/34.23 | 80.40/19.19 |
| | 53.35/23.50 | 97.65/23.50 | 89.50/23.50 | |

17 = Blastogenesis in response to Pokeweed mitogen

Mean/S.E.M.

| Orbit \ Type | d | p | t | |
|--------------|-------------|-------------|-------------|-------------|
| 0 | 74.80/23.78 | 55.10/23.78 | 81.00/23.78 | 70.30/13.73 |
| 1 | 38.60/23.78 | 94.90/23.78 | 88.40/23.78 | 73.97/13.73 |
| | 56.70/16.82 | 75.00/16.82 | 84.70/16.82 | |

18 = Flow cytometry - phycoerythrin controls - % autofluorescence

Mean/S.E.M.

| Orbit \ Type | d | p | t | |
|--------------|------------|------------|------------|------------|
| 0 | 4.98/1.766 | 0.78/1.766 | 2.06/1.766 | 2.61/1.020 |
| 1 | . / . | 1.15/1.766 | 0.72/1.766 | 0.94/1.249 |
| | 4.98/1.766 | 0.97/1.249 | 1.39/1.249 | |

Addendum to Statistical Report

It was suggested by Dr. Surgenor that certain of the measured variables fell into natural clusters, and that it might be helpful to attempt to analyse the influence of the experimental factors on these groups as a whole. The clusters suggested by Dr. Surgenor were analysed using multivariate analysis of variance methods. The SAS procedure GLM was again used, utilizing the MANOVA option.

The first group analysed was a white cell group consisting of the following variables:

| <u>Variable</u> | <u>Table in original report</u> |
|---------------------------|---------------------------------|
| pH | Jacobson 1 |
| pCO ₂ | " |
| pO ₂ | " |
| viability, % FDA positive | Lionetti 1 |
| Phagocytic index 1 | Lionetti 2 |
| Phagocytic index 2 | " |
| Glucose oxidation, PMA | Lionetti 3 |
| Glucose oxidation, FMLP | " |

For this cluster, the orbit factor was not significant at the 5% level ($p = .09$), the bag type factor was significant ($p = .0002$) and there was no significant interaction effect.

The second group was a red cell metabolism group:

| <u>Variable</u> | <u>Table in original report</u> |
|------------------|---------------------------------|
| pH | Jacobson 2 |
| pCO ₂ | " |
| ATP | Jacobson 5 |
| 2,3DPG | " |
| Glucose | " |

For this cluster, the orbit factor was not significant, the bag type factor was significant ($p = .002$) and there was no significant interaction effect.

The third cluster was a red cell structure group:

| <u>Variable</u> | <u>Table in original report</u> |
|---------------------------|---------------------------------|
| Cell Potassium | Jacobson 5 |
| Cell Sodium | " |
| Plasma Potassium | Jacobson 6 |
| Plasma Sodium | " |
| Plasma hemoglobin | " |
| Osmotic fragility | |
| .5%, .55%, and .6% saline | Kevy 1 |
| Phospholipids | Jacobson 13 |
| Cholesterol | " |

None of the factors were significant for this cluster.

The fourth cluster was a red cell membrane binding group:

| <u>Variable</u> | <u>Table in original report</u> |
|-----------------------------|---------------------------------|
| IgG on intact cells | Szymanski 1 |
| IgG inside cells | " |
| C3c on intact cells | " |
| C3c inside cells | " |
| Agglutination with anti-C3a | Szymanski 2 |
| Agglutination with anti-C3c | " |
| Agglutination with anti-C3d | " |
| Agglutination with anti-IgG | " |

None of the factors were significant for this cluster.

The fifth cluster was a platelet metabolism group:

| <u>Variable</u> | <u>Table in original report</u> |
|----------------------|---------------------------------|
| Glucose | Chao 13 |
| pH | Jacobson 3 |
| pCO ₂ | " |
| pO ₂ | " |
| Lactate | Chao 12 |
| Platelet count | Chao 1 |
| Mean platelet volume | Chao 3 |

The orbit factor was significant ($p = .03$) as was bag type ($p = .0002$).

The sixth cluster was a platelet structure group:

| <u>Variable</u> | <u>Table in original report</u> |
|---|---------------------------------|
| * Membrane protein | Kenney 1 |
| * Membrane glycoproteins | " |
| * Cytoskeleton | " |
| Bound IgG | Szymanski 3 |
| Bound C3d | " |
| Bound C3c | " |
| Average transmission electron microscopy score | Ausprunk |

* dropped from the analysis due to missing data.

The orbit factor was significant ($p = .033$) as was the bag type ($p = .048$).

C-4

The final cluster was a platelet function group:

| <u>Variable</u> | <u>Table in original report</u> |
|----------------------------|---------------------------------|
| The above together with: | |
| Aggregation by collagen | Chao 4 |
| Aggregation by ADP | Chao 5 |
| ATP release | Chao 6 |
| Serotonin uptake | Chao 8 |
| β - Thromboglobulin | Chao 10 |
| Thromboxane B ₂ | Chao 11 |

None of the factors were significant for this group.

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JUN 29 1987

PRESIDENT'S OFFICE

June 27, 1987

Dr. Douglas Surgenor
The Center for Blood Research
800 Huntington Avenue
Boston, MA 02115

Dear Doug,

Here is a report on the analysis of the data on position of storage of the samples. As you can see, some of the significance of the orbit or bag type effects change, and there is some effect of position on many but not most of the measured outcomes. I don't know if these effects are consistent or random, but they definitely exist. Taking them into account in the analysis lends somewhat more weight to our conclusions, and it seems obvious that it would be well worth some effort in any future experiments to attempt to dampen these effects, either by designing the storage system to some way reduce the effects and/or by carefully matching on storage position in the design.

I am going to be out of the country during the entire month of July. I hope Nan can clear up any questions you may have until then, and I will be in touch with you when I return.

Sincerely,



Donald K. Blevins, Ph.D.

Statistical Report 3
TPSE

We will discuss in this report, an additional analysis which we have performed on the data from the Initial Blood Storage Experiment. This analysis was an attempt to evaluate the effect if any of the physical location of storage of the blood bags in the storage module.

There were identical storage modules for the orbiter and on Earth. In each of these, the red and white cell bags were stored in three different Dewars, labeled a, b, and c in for the ~~ground~~ ^{flight} unit and correspondingly labeled e, f, and g in the ~~flight~~ ^{ground} unit. Within each Dewar, the bags were stored in one of three positions - front, middle or back (see diagram 1). The platelet bags were all stored in one Dewar, labeled d and h respectively, in each storage unit. Five platelet bags were stored in front and five in back. We therefor introduced for red and white cells two new factors, Dewar and position, and for platelets one new factor, position (front or back). We included these factors together with the previous factors of bag type and orbital status in an analysis of variance as before. Due to the larger number of experimental factors, there was now insufficient data to include any interaction effects, so we used only a main effects model. In the majority of cases, the inclusion of these factors caused no significant change in the conclusions of the previous analyses, but as will be described in the following, in a number of cases one or both of the following effects were noted. First, for some of the experimental variables, the Dewar and/or position in which the sample was stored had a statistically significant effect on the outcome. Second, the inclusion these new factors in the model in some cases changed the significance levels of the original factors. This can occur if the changes in the experimental variable due to the new factors was large enough to partially mask the change caused by the others.

Following is a summary of the new results. Any variables not noted were not significantly changed in this analyses as compared to the previous analysis. (page numbers refer to the previously distributed complete report)

Ausprunk - Transmission Electron Microscopy Data - Red Cells
V1 Total Number of Cells Counted - orbit p = 0.0459

Ausprunk - Transmission Electron Microscopy Data - White Cells
(page 6) V5 Average Ranking - orbit p = 0.0565

Chao 4 (page 12)
V1 Aggregation (by collagen) - orbit p = 0.0202
position p = 0.0195

Chao 5 (page 13)
V1 Aggregation (by ADP) - type p = 0.0634
position p = 0.0206

Chao 6 (page 14)

V1 ATP Release - orbit p = 0.0692
position p = 0.0464

Chao 8 (page 16)

V1 Serotonin Uptake - position p = 0.0506

Chao 13 (page 21)

V1 Glucose - position p = 0.0344

Curby 2 - White Cell Distribution

V1 - Dewar p = 0.0032

V2 - Dewar p = 0.0006

V3 Peak Channel - type p = 0.0049
Dewar p = 0.0049

Jacobson 1 White Blood Cells (page 26)

V1 pH - type p = 0.0001
position p = 0.0001
Dewar p = 0.0577

V2 pCO₂ - orbit p = 0.0001
type p = 0.0001
position p = 0.0016
Dewar p = 0.0044

Jacobson 2 - Red Blood Cells (page 28)

V1 pH - type p = 0.0001
orbit p = 0.0005
position p = 0.0004
Dewar p = 0.0428

V2 pCO₂ - orbit p = 0.0060
type p = 0.0001

Jacobson 3 - Platelets (page 30)

V1 pH - type p = 0.0589
position p = 0.0277

Jacobson 5 - Red Blood Cells (page 33)

V3 Glucose - position p = 0.0359

Jacobson 6 - Red Blood Cells (page 35)

V2 Plasma Potassium - orbit p = 0.0006
position p = 0.0001

V3 Plasma Sodium - position p = 0.0548

Lionetti 1 - White Cells Recovery and Viability (page 42)

V1 Count - position p = 0.0010
Dewar p = 0.0250

V2 % recovery - position p = 0.0010
Dewar p = 0.0249

Lionetti 2 - White Cells Phagocytic Index (page 43)

V1 orbit p = 0.0104
position p = 0.0079
V2 position p = 0.0090

Lionetti 3 - White Cells Glucose Oxidation (page 44)

V1 ^{14}C Glucose Oxidation, Stimulated by PMA -
type p = 0.0068
position p = 0.0370
V2 ^{14}C Glucose Oxidation, Stimulated by FMLP -
type p = 0.0018
orbit p = 0.0005
position p = 0.0764
Dewar p = 0.0114

Szymanski 1 - Red Cells Agglutination (page 45)

V1 IgG on intact cells - position p = 0.0099

Szymanski 2 - Red Cells Agglutination (page 47)

V1 % agglutination with anti-C3a - position = 0.0171
V2 % agglutination with anti-C3c - position = 0.0595

orbit

red white red white red white

| | | | | | | |
|---|----|----|----|----|----|----|
| A | 6 | 22 | 10 | 29 | 20 | 37 |
| B | 8 | 41 | 17 | 26 | 5 | 35 |
| C | 21 | 38 | 3 | 39 | 14 | 23 |
| D | 56 | | 48 | | | |
| | 62 | | 53 | | | |
| | 43 | | 58 | | | |
| | 54 | | 49 | | | |
| | 44 | | 61 | | | |

earth

red white red white red white

| | | | | | | |
|---|----|----|----|----|----|----|
| E | 4 | 40 | 13 | 32 | 15 | 28 |
| F | 11 | 24 | 19 | 30 | 2 | 33 |
| G | 16 | 31 | 1 | 25 | 9 | 36 |
| | 60 | | 50 | | | |
| | 47 | | 98 | | | |
| H | 51 | | 63 | | | |
| | 59 | | 46 | | | |
| | 45 | | 55 | | | |

Diagram 1

EPISTEMOS, INC.

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203 444-0733**

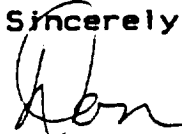
Dr. Douglas Surgenor
The Center for Blood Research
800 Huntington Avenue
Boston, MA 02115

September 8, 1987

Dear Doug;

Here is the analysis of the TOTM platelet bags that Nan said you were interested in. I think it is self explanatory, but as usual feel free to call me if you have questions.

Sincerely



Donald K. Blevins, Ph.D.

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SEP 10 1987

Statistical Report 4

IBSE

We have done an analysis of the effect of microgravity versus controls considering only the platelet bags of type PVC TOTM. Since the only variable factor was the two level earth/orbit factor, a simple t-test suffices for the analysis. The SAS TTEST procedure was used. A basic assumption of the t-test is that the underlying population of each of the two groups are normally distributed with equal variances. If the equal variance assumption is not satisfied an approximate t-test can be used. The SAS procedure TTEST reports both results together with a test statistic for the equal variance assumption. For these data, the assumption of equal variance was statistically acceptable ($p > .10$) except for a few of the outcome variables. Therefore in the following summary, we will report only the results of the standard t-test except for those few items, for which we will report both the standard statistic and the approximate statistic. Unless otherwise noted the degree of freedom for the t-test is 5. The degree of freedom for the approximate t-test depends on the calculated sample variances.

Results

Ausprunk data:

| | |
|------------------|-----------------------|
| Psuedopods | $T = -2.39, p = 0.06$ |
| Aggregated | $T = 0.85, p = 0.44$ |
| Degranulated | $T = 1.20, p = 0.29$ |
| Swollen/Ruptured | $T = 2.07, p = 0.09$ |
| Average Score | $T = 1.66, p = 0.16$ |

Chao data:

| | |
|--|-------------------------|
| Platelet count | $T = 5.16, p = 0.0036$ |
| Hypotonic stress | $T = -1.41, p = 0.22$ |
| Mean volume | $T = 9.07, p = 0.0003$ |
| Aggregation (collagen) | $T = -2.59, p = 0.0489$ |
| Aggregation (ADP) | $T = -1.94, p = 0.11$ |
| (Unequal var. $p = 0.0626$, approx. $T = -1.66, p = 0.23$) | |
| ATP release | $T = -1.38, p = 0.23$ |
| Russell's Viper venom time | $T = 1.20, p = 0.29$ |
| Serotonin uptake | $T = -1.46, p = 0.20$ |
| (Unequal var. $p = 0.0105$, approx. $T = -1.23, p = 0.34$) | |

Serotonin release $T = -1.20, p = 0.29$
 β -Thromboglobulin $T = 1.40, p = 0.22$
 Thromboxane B_2 $T = 1.88, p = 0.12$
 (Unequal var. $p = 0.0025$, approx. $T = 2.22, p = 0.11$)
 Lactate $T = 2.07, p = 0.09$
 Glucose $T = -0.83, p = 0.45$
 (Unequal var. $p = 0.0318$, approx. $T = -0.70, p = 0.55$)

Curby data:

$1-254/ml \times 10^9$ $T = 0.80, p = 0.46$
 $> 254/ml \times 10^9$ $T = -0.19, p = 0.86$
 Peak channel $T = -0.33, p = 0.75$
 Delay factor $T = -1.10, p = 0.32$

Jacobson data:

pH $T = -1.21, p = 0.28$
 (Unequal var. $p = 0.0165$, approx. $T = -1.02, p = 0.41$)
 pCO_2 $T = -2.28, p = 0.07$
 (Unequal var. $p = 0.0001$, approx. $T = -1.91, p = 0.20$)
 pO_2 $T = 2.50, p = 0.0543$

Kenney data:

^{125}I - membrane protein $T = 4.27$ df = 1, $p = 0.14$
 3H - membrane glycoprotein $T = 6.74$ df = 1, $p = 0.09$
 Cytoskeleton $T = 13.28$ df = 1, $p = 0.0479$

Szymanski data:

Bound IgG $T = 1.20$ df = 3, $p = 0.32$
 C3d molecules / platelet $T = 3.03$ df = 3, $p = 0.0560$
 C3c molecules / platelet $T = 3.13$ df = 3, $p = 0.0520$
 Total IgG $T = 3.01$ df = 3, $p = 0.0572$
 Total free IgG in frozen - thawed platelets $T = 2.94$ df = 3, $p = 0.06$